The Effect of Age on the Structure and Composition of the Cell Walls of *Choanephora cucurbitarum*

Craig D. Campbell

(Submitted in partial fulfillment of the requirements for the Masters of Science degree)

Brock University
St. Catharines, Ontario
September, 1980

ABSTRACT

The effect of age on the structure and composition of isolated and purified cell walls from cultures of Choanephora cucurbitarum was investigated by microchemical analyses, visible and infrared spectrophotometry, x-ray diffractometry and electron microscopy. Qualitative evaluation revealed the presence of lipids, proteins, neutral sugars, strong alkali soluble sugars, chitin, chitosan and uronic acids in the cell walls of both the 1 and 7 day old cultures. As the mycelium aged, there was a slight but statistically significant increase in the protein content, and a pronounced rise in the chitin and neutral sugar constituents of the cell walls. Conversely, the decrease in the chitosan content during this period had the net effect of altering the chitin: chitosan ratio from near unity in the younger cultures, to a 2:1 ratio in the 7 day old cell wall samples.

Glutaraldehyde-osmium fixed thin sections of the 1 day old vegetative hyphae of C. curbitarum revealed the presence of a monolayered cell wall, which upon aging became bilayered. Replicas of acid hydrolysed cell walls demonstrated that both the 1 and 7 day old samples possessed an outer layer which was composed of finely granular amorphous material and randomly distributed microfibrils. The deposition of an inner secondary layer composed of parallel oriented microfibrils in the older hypha was correlated with an increase in the chitin content in the cell wall.

The significance of these results with respect to the intimate relationship between composition and structure is discussed.

ACKNOWLEDGEMENTS

I wish to express my gratitude to my long time friend and supervisor, Dr. M. S. Manocha for all the guidance and critical evaluation which led me towards an understanding of true scientific thought. The support of my wife Barbara, was truly infinite throughout my academic career and therefore deserves special recognition in this thesis. I wish also to thank my typist, Mrs. A. Hill for the patience she demonstrated during the final production of this paper.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The traditional view of the cell wall as being a relatively inert structure composed mainly of carbohydrate polymers, has become obscured in light of recent findings. The cell wall is now regarded as a highly complex and dynamic structure which is composed of proteins, lipids and carbohydrates, and is capable of participating in numerous physiological and biochemical activities (Wang and Bartnicki-Garcia, 1970; Manocha and Colvin, 1968). However, our present knowledge concerning the functional significance of these molecules is severely limited, and it is for this reason that the present investigation was initiated. Furthermore, when an adequate amount of data is accumulated on the structure and composition of the cell wall, we will be able to formulate trends which will improve our concept of the mechanism of fungal development and morphogenesis, or host:parasite recognition and specificity (Bracker and Littlefield, 1971; Bartnicki-Garcia and Reyes, 1968).

The composition of the cell wall is only part of the complexity of its structure. The manner in which these components associate in bonding is intimately linked to all other functions of the cell wall. The cell wall may be monolayered or multilayered; it may be amorphous, electron dense, or composed of random, parallel or circular oriented microfibrils (Colvin, 1972). Although the number and structure of fungal cell wall layers is in most cases a species specific phenomenon, a general knowledge of the manner in which they interact is paramount before an understanding of the cell wall's physiology can be achieved

(Hunsley and Burnett, 1970). Similarly, even though there is a great deal of literature published on the changes in the structure of the cell wall under different conditions, little is known of the continuity of change exhibited by both the structure and composition under identical circumstances. The main thrust of this study therefore, was directed towards the understanding of this relationship with respect to aging.

Choanephora cucurbitarum was an ideal candidate for this study because it has been under intense investigation in our laboratory for some time, and therefore its basic physiology is well documented (Devan and Manocha, 1976). Furthermore, given that: (1) C. cucurbitarum develops a secondary cell wall in the older hypha (Letourneau et al., 1976; Manocha and Lee, 1971), (2) in the mycoparasitic system involving the host C. cucurbitarum, and the biotrophic mycoparasite Piptocephalis virginiana, the former becomes resistant to attack upon aging (Manocha and Golesorkhi, 1979), then it seemed apparent that some change in the cell wall was accountable for the development of resistance in C. cucurbitarum. However, it is necessary to point out that no effort was made to positively identify the molecules or macromolecular complexes which are responsible for the resistance phenomenon in this study, but instead lay the groundwork for further investigations.

LITERATURE REVIEW

The fungal cell wall is classically regarded as a rigid structure which protects the fragile protoplast from damage and maintains the characteristic shape of the cell (Rosenberger, 1976). Recent investigations have demonstrated that the cell wall is in fact, a very dynamic structure which is capable of numerous physiological and biochemical activities. A complete comprehension of the complexity of the cell wall must therefore be solved through defining its constituent monomers and understanding how they fit together in the total structure (Rosenberger, 1976). However, the researcher must be cautious when interpreting the data elucidated from these studies, as the endogenous and/or exogenous variables present in the environment can often alter the structure or composition of the cell wall (Huwyler et al., 1979; McMurrough and Rose, 1967).

In order to present a clear picture of the nature of the cell wall, this review is divided into two subsections: (1) composition, and (2) structure of the cell wall. An attempt will be made toward correlating the effects of variables such as culture conditions and age on the structure and composition on the cell wall, and this information is also included in the following subsections.

I Composition of the Cell Wall

A great deal of literature has been published on the composition of the cell walls of vegetative fungal hyphae, particularly after the development of superior mechanical and analytical isolating and evaluating techniques (Letourneau et al., 1976; Rosenberger, 1976). In general, cell wall analyses performed on fungal species from the same genus (Datema et al; 1977a and b; Bartnicki-Garcia, 1968; Bartnicki-Garcia and Reyes, 1968),

from different structures (Bartnicki-Garcia and Reyes, 1964 and 1968), or dimorphic forms of the same species (Sans-Blas et al., 1978; Bartnick-Garcia and Nickerson, 1962) have demonstrated a quantitative difference in their constituent molecules. On the other hand, the evidence indicates that there is very little qualitative variability within the cell wall components of specific classes or orders of fungi. More specifically stated, this qualitative uniformity between fungal groups is such that Bartnicki-Garcia (1968) was able to classify fungi into eight chemical categories (Table I). It is also interesting to note that these divisions are closely correlated to the major classical groupings which were elaborated on morphological criteria and favourably regarded by mycologists in general.

Taxonomic schemes based on the biochemical composition of the cell or cell wall have also been proposed for the prokaryotic kingdom (Rosenberger, 1976; Ghuysen, 1968). It is important to point out however, that assays of this type are not considered as reliable indicators of phylogenetic relationships by some authors. They argue that the cell wall in particular must be regarded as a polyfunctional, complex and metabolically active entity, which is subject to environmental influence (Weijman and Meuzelaar, 1979; Lé John, 1974). Although this controversy is currently unresolved, biological reasoning dictates that further research in this area must be accompanied with the standardization of the cultural conditions, which would therefore in turn, minimize the influence of these factors on the physiology of the microorganism and allow one to draw reliable conclusions.

Approximately 10-15% of the fungal cell dry weight is made up of cell wall material. This percentage appears to be dependent on the fungal species (Moreno, 1969) or morphological form (Bartnicki-Garcia and Nickerson, 1962), and is generally quantified gravimetrically following mechanical iso-

TABLE I. Cell wall taxonomy of fungi. (after Bartnicki-Garcia, 1968)

	Chemical Category	Taxonomic Group	Salient Features
I	Cellulose-Glycogen	*Dictyostelium* *Acetostelium* *Polysphondylium*	sporophores microcyst
II	Cellulose-Glucan	Saprolegniales Peronosporales	vegetative hyphae
III	Cellulose-Chitin	Hyphochytridiomycetes	confirmed in Rhizidiomycetes
IV	Chitin-Chitosan	Zygomycetes	vegetative and reproductive structures
V	Chitin-Glucan	Ascomycetes Basidiomycetes Deuteromycetes Chitridiomycetes	located primarily in mycelial forms
VI	Mannan-Glucan	Ascomycetes Deuteromycetes	notable presence in yeast forms
VII	Mannan-Chitin	Rhodotorulaceae Sporobolomycetaceae	*Rhodotorula* *Sporobolomyces* "pink yeasts"
VIII	Polygalactosamine-Galactan	Trichomycetes	typified in *Amobidium parasiticum*

lation and purification of the cell walls. The absence of cytoplasmic contamination in the isolated cell wall preparations could not be confirmed in the earliest studies because of technical limitations, but with the development of specific cytochemical tests (Sietsma and Wessels, 1976), phase contrast microscopy (Bartnicki-Garcia and Nickerson, 1962) and electron microscopy (Letourneau et al., 1976), homogeneity of the samples has been confirmed. Similarly, it also follows that at least one of these techniques must be employed in all further investigations in order to ensure the validity of the data obtained, because of the inherent differences present in each system (Rosenberger, 1976).

The cell wall is characteristically composed of proteins, lipids and carbohydrates. The latter constituent is the most important since it can make up to 80% of the total cell wall composition,and align into different conformations of monosaccharides or polysaccharides which essentially form the backbone of the structure (Bartnicki-Garcia, 1968). On the other hand, the the protein and lipid moieties are postulated to impart the metabolical capabilities of the cell wall which are necessary for sustaining the viability of the microorganism (Crook and Johnston, 1962; Kreger, 1954).

Proteins in the cell walls of fungi can be firmly or loosely bound in macromolecular complexes, depending on their ultimate function (Rosenberger, 1976). Strong covalently bound protein has been identified as some of the material which fills the interstices of the cell wall reticulum or matrix, and serves towards cementing carbohydrates or lipids into large complexes (Crook and Johnston, 1962). Conversely, the weaker ionically bound polypeptides which can be removed by mild alkali treatments are characteristically assigned enzymatically oriented roles, but this distinction between the two fractions is not without exceptions (Bartnicki-

Garcia, 1968). Cell walls of Neurospora crassa have been shown to contain a discrete protein layer within its ultrastructure, whereas an intimate association of protein and chitin or cellulose microfibrils is evident in the cell walls of Mucor rouxii and Phytophthora parasitica respectively (Hunsley and Burnett, 1970; Bartnicki-Garcia and Nickerson, 1962). Similarly, proteolytic enzyme treatment of some fungal cell walls resulted in a marked change in their gross morphology and ultrastructure, which exemplifies the close relationship between polypeptides and other structural molecules (Hunsley and Burnett, 1970). The proportion of the cell wall that is protein seems to be a species specific phenomenon, as different members of Fusarium are known to contain from 7% to 21% protein in their cell wall dry weight (Barran et al., 1975). This trend is also evident in the dimorphic forms of the same fungus M. rouxii, as demonstrated by Bartnicki-Garcia and Nickerson (1962).

Qualitatively, the acidic nature of the cell wall proteins is attributable to high concentrations of glutamic and aspartic acids in the majority of cases (Letourneau et al., 1976; Nickerson, 1963; Crook and Johnston, 1962). At least 14 different amino acids have been identified in the fungal species studied thus far. However, a notable lack of half-cystine residues in the cell wall fractions is speculated to be indicative of an absence of disulphide bridge formation between the cell layers (Barran et al., 1975). An interesting correlation between the presence of cellulose and hydroxyproline in the cell walls of fungus is also evident in the literature, as this amino acid is otherwise absent in chitin-containing counterparts (Rosenberger, 1976). Finally, qualitative and quantitative comparison of the amino acids in either the total cell, or cell wall proteins rarely demonstrate any substantial differences (Dyke, 1964).

Very little is known about the role of lipids in the cell wall fraction as many researchers initially concluded that they were the result of cytoplasmic contamination. However, this concept was changed through the pioneering work of Dyke (1964) on the cell envelope of Nadsonia elongata. Kreger (1954) postulated that the lipid component confers hydrophobic properties to the surface of the sporangiophore of Phycomyces spp. Electron micrographs of defatted yeast cells revealed the deterioration of their three-dimensional structure and thereby implied an important structural role for lipids in the cell wall (Nickerson, 1963). Considerable stimulation of chitin synthetase activity by crude lipid fractions extracted from the same fungus, suggests the participation of these molecules in a transport or mediatory function in some biochemical pathways (McMurrough et al., 1971). The case in point that becomes obvious by these examples however, is that lipids seem responsible for a wide diversity of functions. The lipid content of the cell wall may reach as high as 25% of the dry weight in some reproductive structures, but is normally reported to be about the mean value of 10% (Letourneau et al., 1976; Bartnicki-Garcia and Reyes, 1968; Nickerson, 1963). Following methylation and hydrolysis of the lipid fraction, its component fatty acids can be analysed by Gas Liquid Chromatography (GLC). Qualitative analysis of the fatty acid methyl esters from the total cell, and cell wall lipid fractions of Choanephora cucurbitarum demonstrate no differences, although they were quantitatively dissimilar. Furthermore, as reflected by their degree of unsaturation, it is apparent that the fatty acids in the whole hypha are more unsaturated then those extracted from the purified cell wall material (Letourneau et al., 1976; Deven and Manocha, 1975; Dyke, 1964). Like their parent molecules, there is a surprising lack of information available concerning the effect of exogenous

factors on the fatty acids in the cell wall. Recent investigations in this area of fungal physiology have just begun to realise the significant role that fatty acids play in stabilising the integrity of the cell wall under environmental stress (Ho, 1979).

The major component of the cell walls of fungal species in general is the carbohydrates. Differential solubility properties of this group of molecules are responsible for their partition into strongly or weakly bound forms, and this is dependent largely on their co-relationship with other molecules in the cell wall (Bartnicki-Garcia, 1968). Further differentiation of the component cell wall carbohydrates has revealed the presence of (1) monosaccharides (Weijman and Muezelaar, 1979), (2) homopolysaccharides (Siestma and Wessels, 1977) (3) heteropolysaccharides (Mahadevan and Tatum, 1967), (4) polyamino sugars (Bartnicki-Garcia and Reyes, 1964 and 1968), and (5) polyacidic sugars (Datema et al., 1977 a). Problems with the identification and characterisation of these macromolecular complexes are compounded by the extraction techniques utilised. For example, drastic acid hydrolysis degrades or destroys pentoses and hexoses in the cell walls of Mucor mucedo (Weijman and Meuzelaar, 1979) or the amino sugars of M. rouxii (Bartnicki-Garcia and Reyes, 1968). Therefore, it is important to fully comprehend the nature of these techniques prior to actual experimentation.

The monosaccharide containing complement of the cell walls of various fungal species exemplify the qualitative and quantitative diversity of these compounds within the members of this group. Of the minor neutral sugars, only mannose has been exclusively detected in the majority of the cell walls of Phycomycetes, Ascomycetes and Basidiomycetes (Siestma and Wessels, 1977; Barran et al., 1975; Wang and Bartnicki-Garcia, 1970), whereas fucose has

been typically found in fungi of the order Mucorales (Weijman and Meuzelaar, 1979; Datema et al., 1977a; Crook and Johnston, 1962). Qualitative variations between cell walls of fungi from the same genus are less dramatic, as the typical composition of Mucor vegetative cell walls is fucose, mannose and galactose (Weijman and Meuzelaar, 1979; Bartnicki-Garcia and Nickerson, 1962). Similar observations were reported for the dimorphic forms of M. rouxii, but quantitative estimates of the carbohydrate composition demonstrated an increase of the mannose content in the yeast form (Bartnicki-Garcia and Nickerson, 1962). On the other hand, there are some exceptions in the literature which illustrate a qualitative dissimilarity in the cell walls between different structures of the same species. In particular, the presence of glucan and melanin and absence of galactose and fucose in the spore cell walls of M. rouxii set this structure apart from the compositions found in its sporangiophore or vegetative counterparts (Bartnicki-Garcia and Reyes, 1964 and 1968; Bartnicki-Garcia and Nickerson, 1962). Unfortunately, this qualitative discrepancy has not been confirmed in any other fungus up to this point.

The polymerisation of long chains of monosaccharides leads to the formation of homopolysaccharides or heteropolysaccharides, depending upon their qualitative content. Since strong alkali treatments readily extract the homopolysaccharide complex from the cell wall, the understanding of the nature of these molecules is clearer than the heteropolysaccharides (Mahadevan and Tatum, 1967). β-1,3 and/or β-1,6 linked glucose sub-units in the form of glucan molecules are found in all fungal classes except probably the Zygomycetes (Siestma and Wessels, 1977; Bartnicki-Garcia, 1968). In high concentrations, this homopolysaccharide along with other structural polymers are believed to be responsible for the integrity of the cell wall. For example, glucan and chitin in Neurospora crassa and Schizophyllum

commune cell walls are intimately associated with the glucan component protecting or surrounding the inner chitinous core (Siestma and Wessels, 1977; Hunsley and Burnett, 1970). Bartnicki-Garcia (1968) postulated that chitosan fulfilled the same structural role in the Zygomycetous fungi as glucan does in the higher fungi. The following sub-section will therefore examine this hypothesis.

The polyamino sugars chitin and chitosan, exlusively form the skeletal basis of the cell walls of Mucoraceous fungi and therefore influence the morphology and physiology of these microorganisms. Chitin, a homopolymer composed of β-1,4 linked straight chains of n-acetylglucosamine, was known to be present in fungi since 1930 because of its ease of isolation, (ie) acid and alkali insoluble (Blumenthal and Roseman, 1957; Frey, 1950). As illustrated in Table I, chitin is not restricted to Mucoraceous fungi, but is also a component in the cell walls of Ascomycetes and Basidiomycetes (Bartnicki-Garcia, 1968). For example, the vegetative hypha and sclerotium cell walls contain 5% and 60% chitin respectively (Rosenberger, 1976). Similarly, the chitin content of the vegetative cell walls of Mucoraceous fungi is just as variable, as M. rouxii, C. cucurbitarum and Phycomyces blakesleeanus contain 9.4, 17.0 and 27% chitin respectively (Van Laere et al., 1977; Letourneau et al., 1976; Bartnicki-Garcia and Nickerson, 1962; Kreger, 1954). Non-conformity in the chitin composition within the cell walls of the reproductive structures or dimorphic forms of the same species in this order is also common (Van Laere et al., 1977). Chitosan on the other hand, is a polycationic amino sugar which is extracted by dilute acetic or nitric acid treatments (Datema et al., 1977b). Composed mainly of glucosamine sub-units, researchers were not able to differentiate this polymer from chitin because of chitin's acid labile acetyl

group, which was normally cleaved during routine hydrolysis of the cell wall (Siestma and Wessels, 1977; Crook and Johnston, 1962). However, specific enzymatic studies coupled with x-ray diffractometry analysis unequivocally identified the presence of chitosan in Mucoraceous cell walls (Tominga and Tsujisaka, 1975 and 1979; Price and Storck, 1975). The importance of chitosan as being a major structural polymer became clear when chitosanase treated cells of M. rouxii and Rhizopus delimar lost their integrity and form (Tominga and Tsujisaki, 1975 and 1979). Quantitative estimates of chitosan in selected members of the order Mucorales have shown it to be a major component in the vegetative hyphae of both C. cucurbitarum and M. rouxii (Letourneau et al., 1976; Bartnicki-Garcia and Nickerson, 1962). Although the chitosan content in the cell walls of the dimorphic forms of M. rouxii is similar, there is significantly less polyglucosamine in the sporangiophore walls of this same fungus (Bartnicki-Garcia and Nickerson, 1962; Bartnicki-Garcia and Reyes, 1964). A possible relationship between the cell shape and polymer composition in these cell walls was suggested.

An understanding of the biosynthetic pathway of chitin and chitosan is paramount to the comprehension of growth, development and morphogenesis in the Zygomycetes. Very briefly, the current hypothesis predicts that N-acetylglucosamine is converted into Uridine diphosphate - N-acetylglucosamine (UDP-N-acetylglucosamine) which is finally aligned into straight chained polymers of chitin by chitin synthetase (McMurrough et al., 1971). Since the optimum for chitin synthetase activity is pH 7.5, it must be compartmentalized in a subcellular fraction. McMurrough et al., (1971) initiated a number of radiotracer studies which demonstrated that the highest chitin synthetase activity was localized in the cell wall fraction of M. rouxii, with substantially smaller amounts of activity located in the mitochondrial

and microsomal fraction of the cells. These findings have also been confirmed in C. cucurbitarum (Manocha and Letourneau, 1978). There is little doubt that N-acetylglucosamine acts as the precursor in this biosynthetic pathway, and it is this fact which has enabled researchers to study cell wall deposition. Labelled N-acetylglucosamine is preferentially distributed at the fungal growing tip outside of the plasma membrane, indicating a close correlation of cell wall deposition with growth and expansion (Colvin, 1972). Furthermore, the biotrophic host: parasite relationship involving C. cucurbitarum and Piptocephalis virginiana respectively, demonstrates that chitin synthetase activity was localized in the sheath zone surrounding the the advancing parasitic haustoria. These data suggested that material similar to that of the host's cell wall was deposited at the host: parasite interface (Manocha and Letourneau, 1978). Unfortunately, the biosynthetic pathway for chitosan synthesis has not been uncovered, although in vitro experiments imply that N-acetylglucosamine is deacetylated prior to the assembly of the chitosan polymer (Storck and Price, 1977).

The final class of carbohydrates that we will consider in this review is the polyacidic sugars. Uronic acids have been detected in only a few fungal cell walls in either a homo- or heteropolymer complex (Bartnicki-Garcia, 1968). A possible explanation for this fact may lie in the technique used for its extraction, as uronic acids are extremely susceptible to decarboxylation or complete destruction by mineral acids (Rosenberger, 1976; Gancedo et al., 1966). Although the mechanism for the synthesis of this compound is not understood in fungi, myoinositol is oxidatively cleaved to form glucuronic acid in some plant cells (Asamizu and Nishi, 1979). The uronic acids are considered to be major building blocks in M. rouxii cell walls, as they can be found in concentrations of up to 25% of the cell wall

dry weight and often complexed with other neutral sugars (Bartnicki-Garcia, 1968). On the other hand, glucuronic acid can be totally absent in some Phycomycetes (Crook and Johnston, 1962), or present in small amounts in the closely related species, C. cucurbitarum (Letourneau et al., 1976). Homopolymers of glucuronic acid (ie) mucoric acid, appear to be ionically bound to chitosan in the cell walls of M. mucedo because: (1) cell wall hydrolysis by nitric acid releases the glucuronan complex, (2) glucuronan could be precipitated by chitosan, and (3) no glucosamine containing polymers were extracted with glucuronic acid by concentrated salt solutions which are specific for ionically bound complexes (Datema et al., 1977a).

Very little is known about the composition of the ash content of the cell wall, which is quantified gravimetrically following intense heating over a long period of time. This fraction characteristically contains the very basic elements used for the synthesis of all molecules in the cell wall, and is generally variable among species and structures (Bartnicki-Garcia, 1968). To illustrate this point, the ash content in the cell walls of the vegetative and sporangiophores of M. rouxii is 18% and 1.7% of the dry weight respectively (Bartnicki-Garcia and Nickerson, 1962).

Thus far, this review has provided an overall picture of the fungal cell wall components, and the following section will try to explain how these compounds fit together to give the final complex structure of the cell wall.

II Structure of the Cell Wall

Under the light microscope, the vegetative fungal cell wall appeared optically homogenous to the earlier microbiologist. Conversely, the cell envelopes of some of the specialized structures such as the zygotes of Phycomyces spp, were differentiated into various layers and were complex in

nature (Burnett, 1970). Initially, these light microscopy investigations were confirmed using techniques such as enzymatic or partial acid hyrolysis, as the cell walls in the treated samples appeared thinner because one or more layers had been stripped off (Potgieter and Alexander, 1965). More recently, direct evidence that the cell walls of fungi in general are differentiated into one or more layers has been achieved through the use of electron microscopy, polarizing optics and heavy metal shadow casting. In general, the chitin or cellulose microfibrils form a complex network with the spaces in the net filled with matrix polymers of various origins (Rosenberger, 1976). Adjacent to this layer, there may be a finely granular amorphous region composed of chitosan, glucan or protein, and which is differentiated from other layers by its texture or staining characteristics (Bracker and Halderson, 1971). Mechanical properties of the cell wall can be modified through altering parameters such as the fibre concentration and the spatial arrangement of other polymers, and these factors do reorganize during growth, development and morphogenesis (Rosenberger, 1976). These characteristics therefore emphasise the plasticity of the structure.

The thickness of the cell wall from a vegetative fungal hypha is typically 0.3mm across (Hunsley and Burnett, 1970; Bartnicki-Garcia and Nickerson, 1962). The formation of the cell wall involves a number of specialized organelles and physiological conditions. The activity of specialized cell wall depositors such as the apical corpuscles in *M. rouxii* (Bartnicki-Garcia and Lippman, 1969) or Spitzenkorper in *Aspergillus* or *Fusarium* spp. (Barron *et al.*, 1975) is usually restricted to the areas of the growing apex, although secondary cell wall formation also occurs in some fungi and is accompanied by depositor activity. The

phenomenon of multilaminate layering has been postulated to be the result of: (1) the cell's inability to incorporate more material into the already formed cell wall, or (2) the need of the microorganism to confer specific properties to its cell wall during growth or differentiation (Hunsley and Burnett, 1970). In electron micrographs, Mucoraceous fungi commonly exhibit a two layered cell wall, whereas the Oomycetous fungi Pythium debaryanum, was reported to contain four layers of electron density (Manocha and Lee, 1971; Manocha and Colvin, 1968; Bartnicki-Garcia and Nickerson, 1962).

The crystalline nature of the microfibril component in fungal cell walls lends itself to easy characterisation. Microfibrils may be composed of cellulose, chitin, mannan or glycoproteins and usually have a diameter of 0.01μ (Rosenberger, 1976 ; Colvin, 1972). The data indicates that chitin is the major building block of these structural elements in Mucoraceous fungi. However, the role of the other common aminopolymer chitosan in microfibrillar development, is not as yet established (Storck and Price, 1977; Bartnicki-Garcia and Lippman, 1969). The fact that microfibrils are specifically orientated in parallel, helical, circular or random configurations in some fungal cell walls, was confirmed by Bracker and Halderson (1971) not to be an artifact of disrupting, extracting or mutilating the sample. In their study, thin tangential cell wall sections of Gibertella persicaria revealed the presence of ordered arrays of microfibrils. Furthermore, heavy metal shadow casting of partially hydrolyzed cell wall preparations have confirmed the presence of all of the above microfibrillar configurations (Colvin, 1972; Manocha and Colvin, 1968). For example, in both C. cucurbitarum and P. debaryanum, the microfibrilar matrix region of their cell walls consisted of a thick random and thin parallel oriented

layers of presumably chitin fibres (Letourneau et al., 1976; Manocha and Colvin, 1968). The phenomena of orientation and microfibril synthesis are intimately related, as spatial and transport limitations both within the cytoplasm and across the plasma membrane dictate that the microfibrils must be synthesized at the cell surface. Present evidence indicates that the plasmalemma is actively engaged in the synthesis of microfibrils, while the cytoplasmic microtubules are responsible for their orientation (Ruiz-Herrera et al., 1975). In algal protoplasts treated with the microtubule formation inhibitors colchicine and isopropyl-N-phenyl carbamate, previously parallel oriented microfibrils became random (Marchant and Hines, 1979). These authors also observed that microtubule bridges between the cell wall and plasma membrane were evident in intact and regenerating algal cells, which seemed to substantiate the microtubule: microfibril relationship.

The nature of the non-fibrillar phases, collectively referred to as the amorphous layers in this review, does not lend itself to extensive and diagnostic x-ray diffractometry or electron microscopy. However, the amorphous layer is an important constituent of the cell wall since it contains proteins, lipids, carbohydrates, glucans and chitosan (Rosenberger, 1976). These macromolecular complexes give this layer the characteristic finely fibrogranular or amorphous appearance as described by many workers, and often can be stripped from the microfibrillar backbone using mild enzymatic or acid hydrolysis techniques (Price and Storck, 1975; Hunsley and Burnett, 1970). Although very little is known about the physical properties that this region imparts to the cell wall, the nature of its components and the fact that it is most often situated as the outermost layer on the cell, implies an important function in transport or in the recognition phenomena (Brian, 1976; Bracker and Littlefield, 1971).

As illustrated in the first section of this review, the composition of the cell wall is truly dynamic in its role in fungal growth, development and morphogenesis. Since cell wall compositional changes are reflected by structural variations, it is not surprising to find gross differences in the framework of the spore, vegetative and sporangiophore cell walls (Bartnicki-Garcia et al., 1968). It is virtually impossible to interpret a rational trend in the structural variations which occur as the spore germinates and grows, as the cell wall may initially act as a carbohydrate reserve and later fulfill a purely supportative role (Morrall and Briggs, 1978). Furthermore, the problem is far more complex in the Phycomycetes, as members in this group characteristically form a vegetative cell wall which is distinct from that of the spores, prior to emergence (Manocha and Lee, 1971; Bartnicki-Garcia, 1968; Hawker, 1966).

The typical spore walls of fungi in the order Mucorales are thick, multilayered and of varying degrees of electron densities (Bracker, 1966; Bartnicki-Garcia and Reyes, 1964; Hawker and Abbott, 1963). Upon germination, the spore wall of M. rouxii becomes thinner and the newly formed vegetative cell wall appears as a monolayered structure composed of randomly arranged microfibrils (Bartnicki-Garcia et al., 1968). Prior to the emergence of the germ tube and formation of the apical dome the spore's cell wall characteristics change, which is demonstrated by its increased sensitivity to chitosanase and deposition of labelled N-acetylglucosamine (Storck and Price, 1977; Bartnicki-Garcia and Lippman, 1969). Similar observations are also evident in the area of the apical zone of the emerging germ tube, which was later characterized as being composed of loosely knit microfibrils in P. debaryanum (Manocha and Colvin, 1968) and C. cucurbitarum (Letourneau et al., 1976). Hunsley and Burnett (1970) noted that the apical fibrils of P. parasitica were smaller

in diameter than their sub-apical counterparts, but similar observations in other species of Phycomycetes have not been reported thus far.

Since an intact microfibrillar mesh has been characteristically observed at the apex of most filamentous fungi, this region has been implicated as the main site for microfibril assembly and deposition. The current hypothesis outlining the organisation of the hyphal apex from the growing tip to the mature hyphae is as follows: (1) Zone α ; a non-extensible region with no apparent cell wall synthesis, (2) Zone β; a region of maximum intussusception where closely spaced transverse microfibrils are assembled, (3) Zone γ ; a highly labile region of multinet fibril development, maximum hyphal extensibility and the area where lateral growth would originate, and (4) Zone δ ; the region of rigidification of the cell wall where development of the multinet matrix is completed, followed by the deposition of a secondary layer composed of longitudinal fibres (Burnett, 1970). Electron micrographs of preferentially treated hyphal apicies have thus far confirmed the organisational trends predicted by this hypothesis (Hunsley and Burnett, 1970).

Our understanding of the changes which occur in the cell wall structure over a prolonged period of time is very limited. In general, the young cell wall has been regarded as a monolayered homogenous structure, smooth in appearance and closely appressed to the plasma membrane (Manocha and Lee, 1971). On the other hand, ultrastructural studies of the older cell wall demonstrated the appearance of one or more additional layers which are morphologically distinct from each other (Letourneau et al., 1976). These changes are paralleled by the sensitivities of the cell walls to enzyme hydrolysis (Price and Storck, 1975) or antigen labelling studies. In this later case, increases in antigen binding to the older cell walls of

Allomyces was postulated to be due to the degeneration of the outer layer, which therefore exposed the underlying consituents (Fultz and Sussman, 1966). This hypothesis was supported by earlier work carried out by Aronson and Machlis (1959), who reported a 50% increase in the chitin microfibril content over a 70 hr. time period. Secondary cell wall formation in plant cells is a relatively wide spread phenomenon. The deposition of an cellulose layer in 60 day old cotton plant cells is correlated with a decrease in protein and an increase in glucose and cellulose residues in the total cell wall (Huwyler et al., 1979). Alternately, deposition and therefore the structure of the cell walls of P. debaryanum, did not vary significantly between 5 and 35 days of incubation (Manocha and Colvin, 1968). Clearly, in order to thoroughly evaluate the effect of age on fungal cell walls, an attempt must be made to standardize environmental factors which ultimately affect growth. This was not apparent in any of the studies reviewed to this date.

Fungal morphogenesis is accompanied by, and probably dependent upon specific alterations of the chemical structure of the cell wall (Bartnicki-Garcia, 1968). Although the production of the vegetative or yeast-form of a dimorphic species is dependent on the environmental conditions, it is the resulting biochemical and physiological responses which dictate the final morphology. This response is illustrated by the fact that cell wall synthesis is apically located in the hyphal form and dispersed uniformly around the perimeter of the yeast cell (Bartnicki-Garcia and Lippman, 1969). Even though there is a continuity in the structural elements between the two forms, difference in the manner in which they're assembled seems to account for the observed dimorphism (Bartnicki-Garcia et al., 1968). Characteristically, the yeast forms of most fungi including

M. rouxii and Paracoccoides brasiliensis, possess a thick, bilayered cell wall which is composed of an outer fibrillar and inner amorphous region. In contrast to this, the filamentous cell walls are constructed of a solid network of microfibrils (Moreno, 1969; Bartnicki-Garcia and Nickerson, 1962). Unfortunately, there is little evidence concerning the control mechanisms responsible for the dimorphic nature of these microorganisms. However, it is speculated that the activity of an internal inducer of "yeast-like" development permits a uniform cell wall deposition which induces the formation of a spherical cell (Bartnicki-Garcia, 1963). The resulting structure in turn, maintains the integrity of the cell against environmental stress.

It has been the intent of this review to familiarize the reader with the multiple aspects of the structure and composition of the fungal cell wall. There is no doubt that the innate complexity of this structure lends itself to a great deal of speculation in the nature of its function. However, perhaps the most important point which is expressed throughout this review is the intimate association between structure and composition. This study attempts to bring this association into a clearer prospective.

MATERIALS AND METHODS

I Organism and Cultural Conditions

Cultures of Choanephora cucurbitarum (Berk. and Rav.) Thaxter were routinely maintained on a solid medium consisting of malt extract, 20g; yeast extract, 2g; and agar, 2g (all of Difco trademark); in one litre of distilled water. After adjusting the pH to 6.5 with either 1N NaOH or 1N HCl, the culture medium was autoclaved and inoculated with fragments of mycelium. Following incubation of the cultures at 23°C (± 1°C) (Percival, Iowa, USA) for 4 days, the spores were collected in sterile distilled water. A standardised spore suspension consisting of 3.0×10^9 spores/ml was used throughout this study to inoculate the liquid medium (same medium as above minus agar) and to obtain the fungal mycelia for the cell wall preparations.

II Measurement of Growth of Choanephora cucurbitarum

The growth of C. cucurbitarum was measured quantitatively on a mycelial dry weight basis over an 8 day period of incubation, with samples taken at regular intervals.

Five millilitres of the standardised spore suspension was added into 200 ml of the liquid medium contained in 500 ml Erlenmyer flasks. The cultures were incubated at 25°C (± 1°C) on a Gyrotary Water Bath Shaker (New Brunswick Scientific, New Jersey, USA) at a speed of 100 revolutions per minute. At 24 hr. time intervals, the mycelia from each flask was harvested on a preweighed Millipore filter (47 mm diameter, 1.2 M pore size), washed with distilled water until the elutant appeared clear, and dried overnight at 60°C in an oven to a constant weight. The mycelial dry weight was then measured gravimetrically.

III Cell Wall Isolation and Purification

All isolation and purification procedures were carried out at $4^{o}C$ to prevent denaturation of the cellular components. The cell wall material which was used throughout this study was extracted from C. cucurbitarum cultures grown in the same manner as outlined previously. The mycelium from the 1 and 7 day old cultures was harvested on a coarse sintered glass funnel and washed repeatedly with cold distilled water to remove all traces of culture medium. The sample was homogenized at full speed in an Omni-Mixer (Sorvall Inc., Connecticut, USA) for 10 minutes and centrifuged at 3000 x g (IEC - B20, Centrifuge, International Equipment Co., Massachusetts, USA). After discarding the supernate, the pellet was resuspended in 40 ml of cold distilled water and treated in a French Pressure Cell Press (American Instrument Co. Inc., Maryland, USA) at 16,000 lbs./inch^2. Following subsequent recentrifugation, the pellet was resuspended as before and sonicated with a Sonicator Cell Disruptor (3/4" tip, Heat Systems-Ultra Sonic Ltd., New York, USA) at 60% amplitude and at 60% of the duty cycle for 10 minutes, in order to prevent excessive heat build-up. The sonication and centrifugation steps were repeated numerous times until the supernate appeared completely clear. The sonication treatments are extremely important in cell wall isolation because the cavitation generated during this procedure significantly reduces the viscosity of the cytoplasm, which facilitates its release into the supernate (Manocha and Colvin, 1967).

Purity of the cell wall fraction was confirmed using the light (Leitz, Orthoplan) and electron microscope (Phillips EM 300, Phillips Electronics, The Netherlands). In the former technique, a drop of Lugol's Iodine solution stained cytoplasmic contamination bright yellow whereas

cleaned cell wall material appeared pink to violet under the light microscope (Bartnicki-Garcia and Nickerson, 1962). Cell wall material which was used for analytical purposes was lyophilized and stored at -4°C until required. Isolated cell walls which were used in the ultrastructural studies were fixed immediately for thin sectioning, or dried on cleaned glass slides after acid treatment for replica preparations as described by Manocha and Colvin (1967).

IV Analytical Procedures

(A) Extraction of Lipids

Lipids were extracted from preweighed samples of lyophilized cell wall material of C. cucurbitarum using two different techniques. The first procedure utilising the Soxhlett Extraction apparatus was preferentially used due to the increased ease of recovery of the defatted cell wall material following extraction. In this technique, the sample was refluxed with 125 ml of petroleum ether (J.T. Baker Chemical Company, New Jersey, USA) for 16 hr., followed by chloroform:methanol (1:2 v/v) for 8 hr. (Bartnicki-Garcia and Nickerson, 1962). The supernates were combined and the solvents were removed in vacuo with a Büchi 'Rotovapor R' (Büchi Inc., Switzerland). The cell wall lipids were quantified gravimetrically.

The second technique involved the maceration of the cell wall material three times with 20 volumes of chloroform:methanol (2:1, 1:1, 1:2, v/v). The supernate was removed after each treatment by filtering the homogenate through a No. 1 Whattman defatted filter paper into a separatory funnel. After combining the three extracts, exogenous material was removed from the lipid phase with 0.9% NaCl according to the methods of Folch et al. (1957) and Bligh and Dyer (1959). The chloroform and

methanol were removed *in vacuo* and the lipids were collected and weighed.

Lipid material that is strongly bound in macromolecular complexes in the cell wall and was not removed by the previous treatments, was extracted with a 10% methanolic potassium hydroxide solution as described by Safe (1974).

Lipid material was routinely stored at -4°C in benzene:amyl alcohol: chloroform (1:1:1 v/v) with 0.01% butylated hydroxytoluene added to prevent auto-oxidation. Fatty acid methyl esters of the lipid fractions were prepared following treatment with petroleum ether:anhydrous acetone: 0.5 M sodium methylate (1:1:5). After hydrolysis with 0.5 N HCl, the upper phase containing the fatty acid methyl esters was dried under nitrogen and injected into the gas-liquid chromatograph (GLC).

The GLC used for all analysis was a Hewlett Packard Model 5700A, equipped with a flame ionization detector and a Fischer Recordall 5000 Recorder Integrator. The oven was programmed to increase in temperature from 140°C to 200°C at a rate of 2°C/minute. The glass column (180 cm x 3 mm) was packed with 10% Silar 10 C (Apolar 10 C, Applied Sciences, Michigan, USA) on Chromosorb Q AWDMCS, 100-120 mesh size. Comparison with authentic standards (Serdary Research Laboratories Inc., London, Canada) yielded the qualitative identification of the fatty acid methyl ester profiles evident in the experimental samples, whereas the area under each peak gave a quantitative estimate of the percent composition of the particular fatty acid.

(B) Extraction of Protein

One and seven day defatted cell walls of *C. cucurbitarum* were extracted with either hot 1N NaOH or cold 5% trichloroacetic acid (TCA). In the later technique, 50 mg of sample was homogenized in 100 volumes

of 5% TCA at 4°C. Following centrifugation at 3000 x g for 10 minutes, the supernate was discarded and the proteins in the pellet were resolubilised in 1N NaOH and recentrifuged. The supernates were combined after the insoluble cell wall material was washed several times with cold distilled water.

In the former technique 50 mg of the cell wall was treated with 100 volumes of 1N NaOH at 100°C for 1 hour in closed teflon stoppered test tubes. Following the removal of the supernate, the pellet was washed with distilled water as before, and retreated with the hot alkali.

An aliquot of the extracts from each of the preceding treatments was used for the quantitative protein determinations. Protein concentrations in the cell walls of C. cucurbitarum were estimated by the method of Lowry et al. (1951) or the Biuret (Gornal et al., 1949) colorimetric reaction, using Bovine Serum Albumin Fraction V (Sigma Chemical Company Inc., Montana, USA) as the standard. Due to the inadequate sensitivity of the latter technique however, the Lowry Method was routinely used in this investigation.

The presence of proteins in subsequent extractions of the cell wall with 1N acetic acid and 30% potassium hydroxide solutions was also examined in order to assure their complete removal by the previous techniques.

Finally, the amino acid composition of the solubilised proteins was determined following 6N HCl hydrolysis for 20 hr and subsequent analysis with an Amino Acid Analyser at the University of Toronto.

(C) Extraction of Carbohydrates

Fifty milligrams of defatted cell walls from 1 and 7 day old cultures of C. cucurbitarum were treated initially with 1N NaOH (100°C, 1 hr.), then with 1N acetic acid (90°C, 2 hr.) and finally with 30% KOH (100°C, 1 hr.).

After each treatment, the pellet was washed several times with distilled water and retreated with each of the reagents. The 30% KOH extract was desalted with a Sephadex G-10 column (45 cm x 2.5 cm) (Pharmacia Fine Chemicals, Sweden) prior to further analysis. Each sample was brought to a known volume with distilled water, and aliquots from these were quantified for the presence of neutral sugars utilizing the Anthrone Method (Umbreit et al., 1964) with glucose as the standard.

Fractionation of the components extracted by each of the above solvents was preceded by repeated hydrolysis of the ethanol precipitated sugars with 1N HCl (100°C, 1 hr., under N_2) in sealed test tubes. Separation of the hydrolysates into a neutral sugar, amino sugar, uronic acid and amphoteric fraction was facilitated by cation (Dowex 50 x 8 - 400, H^+ form) and anion (Dowex 1 x 8 - 400, CH_3COO^- form) (Sigma Chemical Company, Montana, USA) exchange chromatography (Van Laere et al., 1977).

The neutral and amino sugar components from the cell walls were first converted into their corresponding alditol acetates before being injected into the GLC according to the procedure of Stradler (1976). The oven of the GLC was programmed for a 4 minute initial hold at 180°C, followed by a 2°C/minute linear increase in temperature to 240°C. The glass capillary columns used for the subsequent separation and qualitative identification of the components of the neutral and amino sugar fractions were: (1) neutral sugars; a 180 cm x 2 mm column packed with 3% SP-2330 on 100-120 Supelcoat, and (2) amino sugars; a 90 cm x 2 mm column packed with 3% SP-2340 on 100-120 Supelcoat (Supelcoat Inc., Pennsylvania, USA). Co-chromatography with authentic standards yielded the necessary qualitative data whereas the quantification of the sugars was facilitated through the use of an internal standard. Since preliminary studies failed to detect the presence of ribose in the cell wall of C. cucurbitarum, this sugar was

used as the internal standard.

Uronic acids in the eluted fractions were quantified by the Carbazole Reaction as modified by Bitter and Muir (1962) with glucuronic acid as the standard. The presence of amphoteric components in these samples were detected solely through the use of gravimetric techniques.

An attempt to detect the presence of homopolysaccharides in the form of glucans or mannans was made in the 30% KOH extract through their precipitation with Fehling's Reagent (British Drug House, Toronto, Canada). The characteristic red, brown or yellow precipitate of the cuprous-oxide: homopolysaccharide complex formed during the reaction with this reagent was quantified gravimetrically (Dawson et al., 1969). Due to the apparent lack of any substantial quantities of these compounds in the cell walls of C. cucurbitarum, the sensitivity of the gravimetric technique prevented the elucidation of reliable results.

(D) Extraction of Amino Sugars

Ten milligrams of cell wall from C. cucurbitarum were hydrolyzed in a solution containing H_2O:2N $NaNO_2$:2N HCl (1:0.5:1.5, adjusted to pH 3.0) for 1.5 hr. at room temperature. Filtered air was blown across the surface of the samples for 15 minutes to remove any excess nitrous oxide after the test tubes were uncapped. Following centrifugation, the supernate (containing 2,5-anhydromannose, polyglucuronic acid, heteroglucans and phosphate) was loaded onto the Sephadex G-10 column and eluted with 90 ml of distilled water. An aliquot of the elutant was used for the detection of uronic acids (Bitter and Muir, 1962). The pellet (containing chitin) and a sample of the untreated cell wall (containing both chitin and chitosan) were hydrolyzed in 6N HCl for 5 and 16 hr. in a N_2atmosphere (Datema et al., 1977b). After centrifugation and washing the residue, the supernates

were combined and analyzed for the quantification of the amino sugars by the Elson-Morgan Reaction (Johnson, 1971). The difference in the glucosamine concentration between the nitrous acid treated and the untreated sample was considered representative of the chitosan content in the cell wall (Datema et al., 1977b).

(E) Chitin Determination

In order to ascertain the purity of the chitin isolated by the nitrous acid technique, the treated cell wall sample and an authentic chitin standard (Sigma Chemical Company, Montana, USA) were subjected to infared spectroscopy (Perkin-Elmer Company Inc., Massachusettes, USA) and X-ray diffractometry (Phillips, The Netherlands). The crystallinity of chitin enabled the use of these techniques for the positive characterisation of this compound (Bartnicki-Garcia, 1968).

(F) Ash Determination

Lyophilized cell walls were weighed and heated at 700^{o}C for 6 hr. in a muffle furnace (Letourneau, 1978). After cooling, the ash content of the cell walls was determined gravimetrically.

V Electron Microscopy Techniques

(A) General Morphology

Mycelium and purified cell wall material from 1 and 7 day old cultures of C.cucurbitarum were fixed in a cold 0.2 M sodium phosphate buffer (pH 6.5) containing 3% glutaraldehyde for 2 hr. in vacuo. After discarding the supernate, the samples were post-fixed for 1.5 hr. in 1% osmium tetroxide in buffer and then dehydrated with an ethanol and propylene oxide series (Manocha and Colvin, 1967). The specimens were embedded in Spurr's Mixture (Spurr, 1969), and thin sections were cut using glass knives on a Reichert Ultramicrotome UM3 (Austria). Sections were stained

with lead citrate according to the methods of Reynolds (1963), and examined under the electron microscope (Phillips EM 300, Phillips, The Netherlands).

(B) Cell Wall Structure

In order to examine the texture of the different cell wall layers, purified material was partially hydrolyzed in 1N HCl for various periods of time at 100°C. Treated or untreated samples were layered on clean glass microscope slides and dipped in a 1,2-dichloroethane solution containing 0.4% Formvar (E.F. Fuller Inc., New York, USA). The replicas were floated off the slides in a 10% ammonium hydroxide solution, and after 4 hr. the solution was neutralised by repeated flushings with distilled water (Manocha and Colvin, 1967). The formvar replicas were mounted on copper grids and dried overnight in a dessicator. A Pd-Au alloy film was deposited on the grids at a 15°angle in a Vacuum Evaporator (Varian, USA), and the metal shadowed replicas were examined under the electron microscope.

RESULTS

I Measurement of the Growth of Choanephora cucurbitarum

The aim of this growth experiment was to select two incubation periods which would be representative of the young and old hyphae of Choanephora cucurbitarum. Cultures of this fungi were therefore grown under a set of defined environmental conditions which were used throughout this investigation. Subsequently, the growth curve was constructed after the total mycelium from each of cultures was harvested by vacuum filtration, thoroughly washed and dried to a constant weight. This technique was used since previous reports indicate that this procedure yields the best estimate of fungal growth on liquid medium (Lilly and Barnett, 1951).

The data reveals that the growth of C. cucurbitarum is linear during the first five days of incubation (Figure 1). The apparent lack of a lag phase in the growth of this fungus is probably a consequence of the sampling interval which was used (Mandels, 1965). Following the linear increase in the weight of the total mycelium, the growth rate of C. cucurbitarum decreases and results in the levelling off of the growth curve after the sixth day of incubation (Figure 1). The cultures maintained this stationary phase of growth throughout the remainder of the experiment.

The culture filtrate pH is a useful indicator of the proportional utilization of carbon and nitrogen by the fungus and is often linked to specific physiological changes which occur during growth (Moore-Landecker, 1972; Lilly, 1965). The data illustrates that a correlation exists between the rate of growth of C. cucurbitarum and the culture filtrate pH.

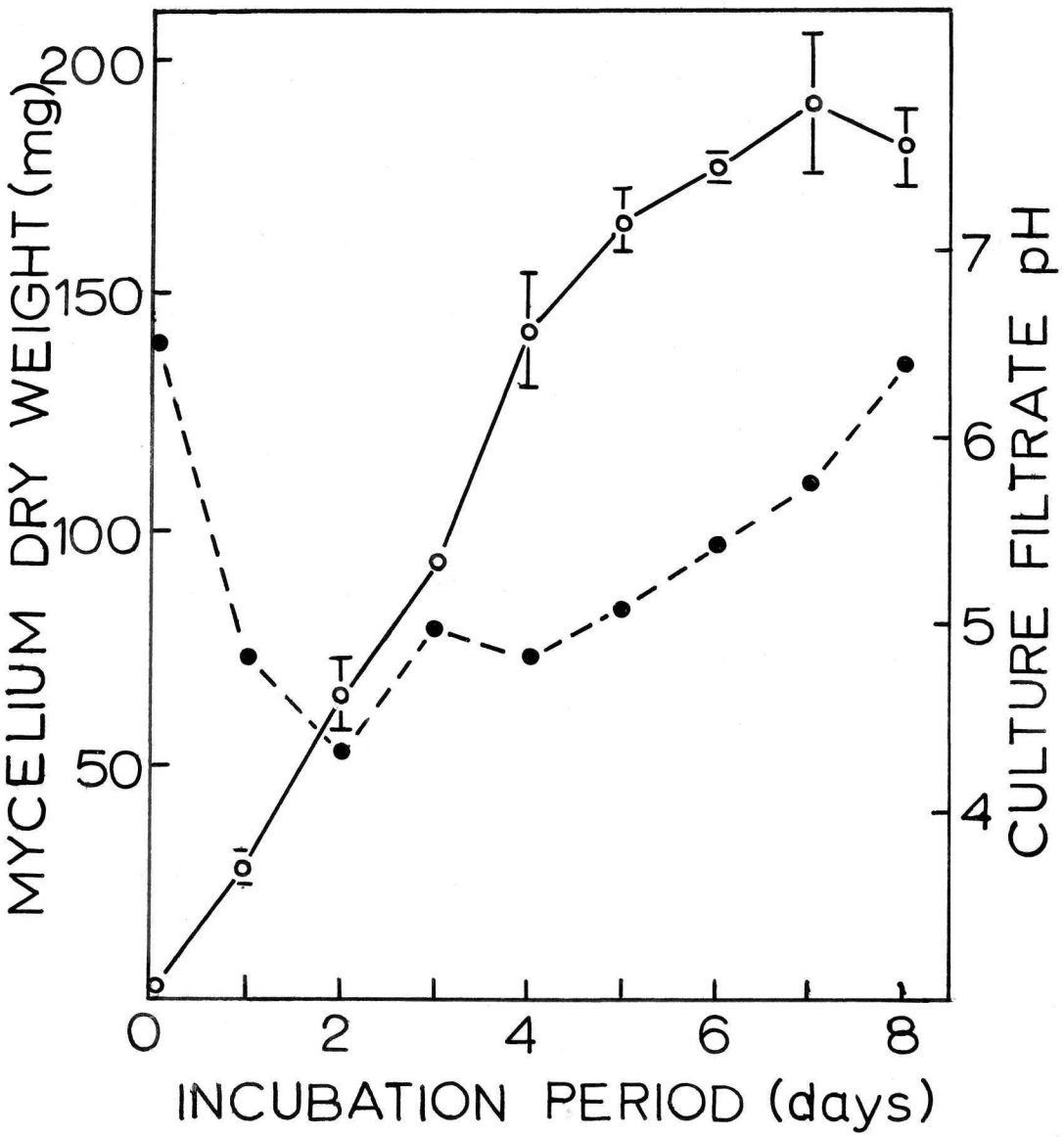
TABLE II. The growth of Choanephora cucurbitarum on liquid medium.

Incubation Period (days)	Mycelium Dry Wt (mg)	Culture Filtrate pH
0 (spores)	4.5 (0.35)	6.5
1	28.6 (2.85)	4.8
2	65.6 (3.19)	4.4
3	92.3 (0.35)	5.0
4	142.4 (6.41)	4.8
5	166.7 (3.40)	5.1
6	176.8 (1.04)	5.4
7	191.6 (6.93)	5.8
8	181.5 (3.75)	6.4

Note: Figures in brackets refer to the standard deviation of the mean.

Culture conditions for this growth study are described under Materials and Methods.

FIGURE 1 The effect of age on the growth and culture filtrate pH of Choanephora cucurbitarum.



Note: Bars represent the standard deviation of the mean.

The hydrogen ion concentration of the medium is always higher in the actively growing cultures and lower in the stationary phase (Figure 1). However, no attempts to regulate or assess the effect of pH on the growth of this fungus were made in this investigation.

Routine light microscopic observations of any of the growing cultures failed to reveal the presence of a significant number of ungerminated spores or reproductive structures. This observation is particularly important in light of the nature of the following experiments. Published reports on the vegetative, spore or sporangiophore structures from the same fungal species demonstrate their uniqueness in both composition and structure (Bartnicki-Garcia and Reyes, 1964 and 1968; Bartnicki-Garcia and Nickerson, 1962). Therefore, a heterogenous cell wall mixture in this study would have resulted in the elucidation of unreliable data. Since there is an intimate link between the environment and the production of reproductive structures in fungi, it appears that the cultural conditions which were selected for this investigation are responsible for the poliferation of the vegetative hyphae of C. cucurbitarum (Barnett and Lilly, 1950).

The standard deviation which has been calculated from at least 6 replicas per incubation period is presented in Table II and illustrated in Figure 1. Due to the inherent limitations of the gravimetric technique, average deviations of 3.0 mg are common within this growth study. This fact was considered during the interpretation of this data.

The 1 day old sampling period was selected for further investigative analyses of the young cell wall because it was the earliest time that enough mycelium could be harvested. Alternately, the 7 day old cultures were representative of the period of transition between the linear and

stationary periods of growth, which is the point where cultural parameters become limiting (Moore-Landecker, 1972; Lilly, 1965). These decisions were also influenced by the previous findings of Letourneau et al. (1976) and Manocha and Lee (1971). These authors reported that the young cell wall of C. cucurbitarum was monolayered, whereas the 5 day old vegetative hypha possessed a bilayered cell wall.

II Composition of the Cell Wall

The first prerequisite to any biochemical analysis is the purity of the cell wall preparation. Purity of the isolated cell wall fractions used in this study was confirmed by both histoplasmic and electron microscopic techniques (Figures 2a and 2b). In the latter instance, the conspicuous lack of cytoplasmic contaminants in the thin sections, or plasma membrane adhering to the cell wall is indicative of a pure preparation.

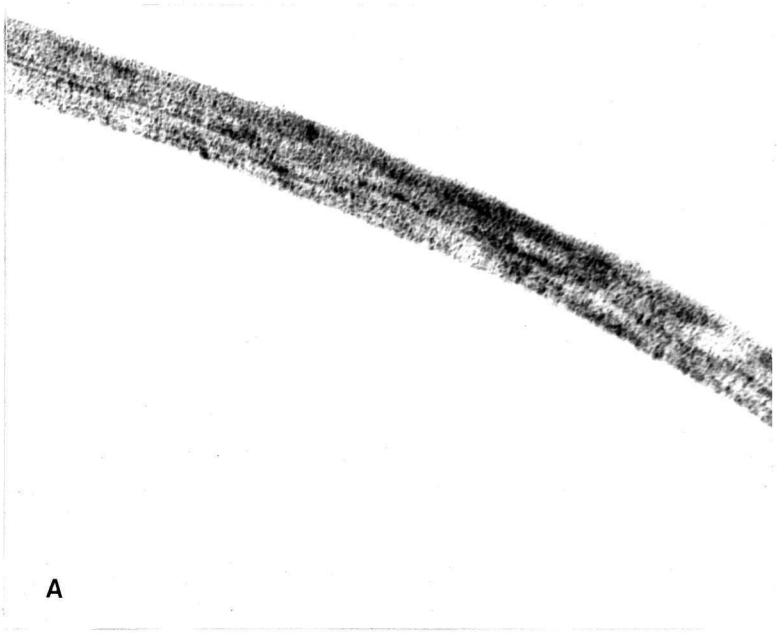
The elucidation of a reliable value of the percentage of the cell which is made up of cell wall is limited by the gravimetric technique. The data indicates that the 1 and 7 day old cells of C. cucurbitarum contain 17.9% and 17.6% cell wall respectively. There does not appear to be a significant difference in the amount of cell wall which was recovered from either of these samples. This observation was confirmed by statistical analysis at the 95% confidence interval (Appendix I).

(A) Lipids

In practice, the lipids within the cell wall are extracted before all other constituents because of their ease of solubility in chloroform, methanol or petroleum either. In this investigation, two techniques for removing the loosely-bound lipids of the cell wall were employed in order to ensure their complete extraction. Neither method demonstrated the

Figure 2(a) Transmission electron micrograph of the cleaned cell wall preparation from the 1 day old cultures of Choanephora cucurbitarum (x64,200).

Figure 2(b) Cytoplasmic free preparations from the 7 day old samples (x64,200).



A

B

TABLE III. The effect of age on the cell wall composition of *Choanephora cucurbitarum*

Components (% dry wt. cell wall)	Incubation Period (days) 1	7
Protein	16.3 (0.77)	19.3 (0.59)*
Lipid	6.1 (0.01)	8.1 (0.41)***
Neutral Sugars	20.4 (0.81)	26.4 (1.33)*
Strong Alkali Soluble Sugars	7.7 (0.25)	3.5 (0.19)*
Chitin	16.9 (0.19)	21.3 (0.72)***
Chitosan	18.4 (0.98)	10.0 (0.95)*
Uronic Acids	1.1 (0.11)	1.1 (0.12)**
Ash	5.7 (0.20)	3.9 (0.11)**
Total	92.6	93.6

Note: Figures in brackets refer to the standard deviation of the mean.

Results of the students t-test($P<.05$):

Appendix I; comparing the 1 and 7 day samples are denoted by:

* Significantly different

** Not significantly different

*** Assumptions of the test could not be fulfilled.

ability to preferentially extract more or qualitatively different lipid from the cell wall samples. However, the Soxhlett apparatus was routinely used since the cell wall yield from this technique was superior. Further attempts to remove and characterize any lipid which was tightly-bound within the cell wall was carried out using a 10% methanolic potassium hydroxide solution (Safe, 1974). Due to the small quantity of this type of lipid in these samples, the gravimetric technique was unable to detect their presence. However, it is important to note that upon methylation and hydrolysis of the residue which remained after the alkali extract was reduced, fatty acids were detected. This observation is believed to be a consequence of the superior resolution inherent in the Gas-Liquid Chromatography (GLC) technique. Finally, the resulting chromatograms of the fatty acid profiles from the loosely or tightly-bound lipid fractions appeared to be identical.

The data reveal that the lipid content of the cell walls of C. cucurbitarum is approximately 7% of the dry weight (Table III). There does not seem to be any quantitative difference between the 1 and 7 day old samples, but this observation could not be confirmed statistically because the assumptions of the test could not be fulfilled. In particular, the calculated variances of this data were significantly different (Appendix I).

The qualitative fatty acid composition of the total cell wall lipids which are common to both incubation periods are as follows. The fatty acids in decreasing order of abundance are: oleic ($C_{18:1}^{\Delta 9}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$) and palmitoleic acid ($C_{16:1}^{\Delta 9}$). The data also reveals the presence of smaller amounts of myristic ($C_{14:0}$) and lauric acid ($C_{12:0}$) (Table IV). The appearance of γ-linolenic

($C_{\gamma 18:3}^{\Delta 6,9,12}$) and linoleic acid ($C_{18:2}^{\Delta 9,12}$) in the 7 day old cell wall lipid was accompanied by a decrease in the concentration of all fatty acids except stearic acid, whose % composition did not change within the sampling periods (Figure 3).

The degree of unsaturation (Δ/mole) has been calculated for each incubation period and is presented in Table IV. Age appears to have a significant effect on the number of unsaturated bonds in the fatty acid hydrocarbon chains in this study. This point is notable since the degree of unsaturation in the 1 and 7 day old samples were 0.4651 and 1.268 Δ/mole respectively. These values also differ from those which have been previously reported for the cell wall or total lipids of C. cucurbitarum (Letourneau et al., 1976; Devan and Manocha, 1976).

The variation of the experimental data about the mean fatty acid % composition values was approximately 3% (Table IV). Although this figure may seem high considering at least 3 replicas of each experimental condition were run, the standard deviations are within the limits inherent in this technique.

(B) Proteins

Protein was extracted from the defatted cell walls of C. cucurbitarum with either hot NaoH or cold Trichloroacetic Acid (TCA). Neither technique demonstrated an ability to preferentially extract more protein than the other. Similarly, re-extraction of the samples with these solvents usually yielded an additional 1 - 2% of the cell wall dry weight of protein, and this amount is included in the cited values. The further treatment of the cell walls with 1N acetic acid or 30% KOH also extracted a small quantity of protein. It is postulated that these polypeptides were closely associated with other molecules which were released by these

TABLE IV: Fatty acid analyses of the lipids extracted from the cell walls of Choanephora cucurbitarum

Incubation Period(days)	Fatty Acid Percent Composition								Degree of Unsaturation* (Δ/mole)
	12.0	14.0	16.0	16.1	18.0	18.1	18.2	γ 18.3	
1	7.5 (0.4)	9.7 (0.45)	20.7 (1.23)	11.4 (2.63)	15.7 (1.17)	35.1 (1.90)	t	ND	0.4651
7	1.7 (0.91)	3.7 (1.15)	12.0 (1.12)	9.0 (0.87)	14.4 (4.92)	21.0 (0.59)	17.8 (2.02)	20.4 (4.22)	1.268

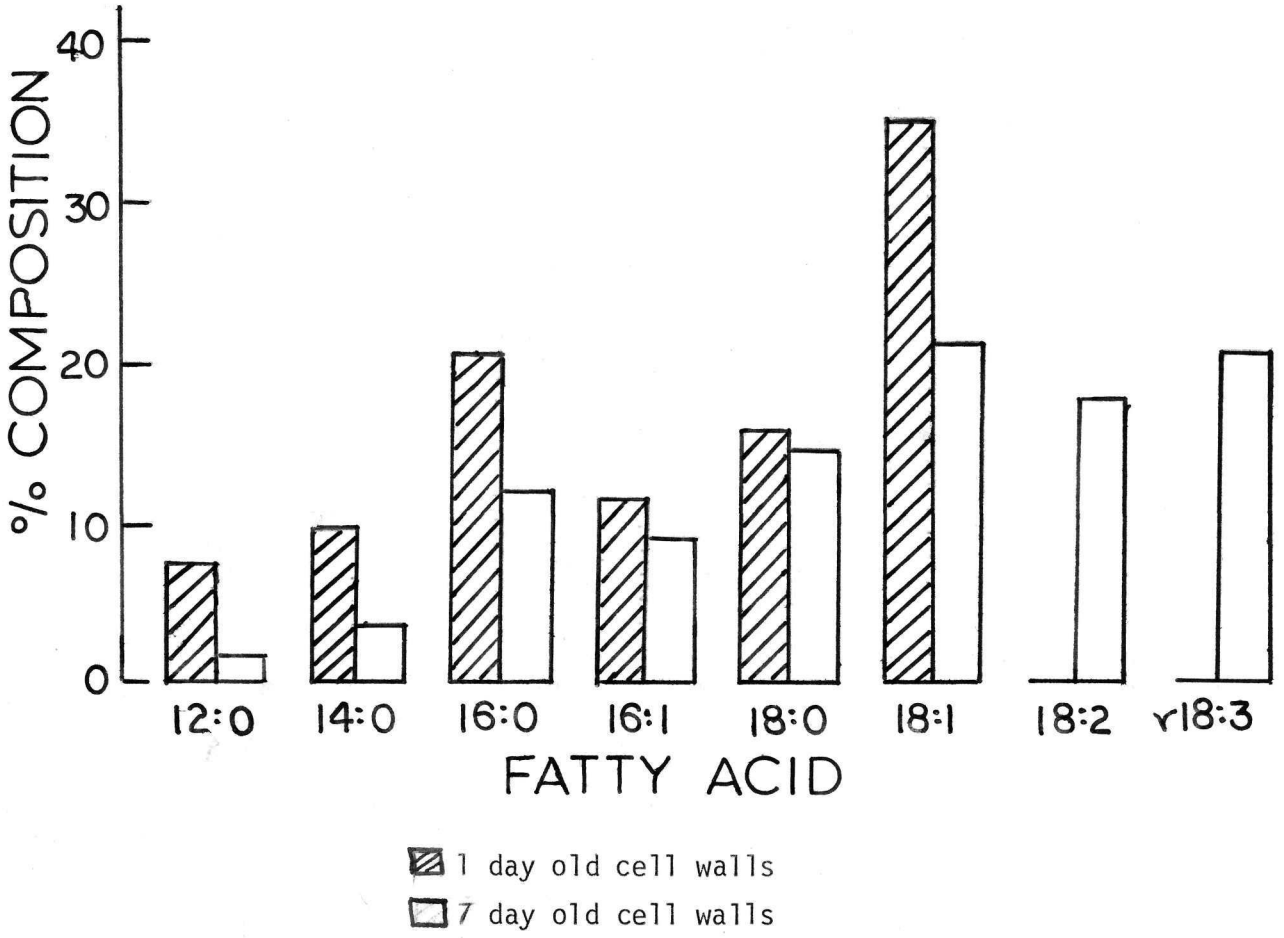
Note: Figures in brackets refer to the standard deviation of the mean.

ND = Not detected

t = trace

* Degree of unsaturation = 1.0 x (% monenes ÷ 100) + 2.0 x (% dienes ÷ 100) + 3.0 x (% trienes ÷ 100)

FIGURE 3 The effect of age on the fatty acid composition of the cell walls of Choanephora cucurbitarum



reagents. Finally, when the amount of protein which was extractable by the above techniques was summed, it became clear that there was a significantly higher proportion of protein in the older cell wall as opposed to the younger cell wall (Table III, Appendix I).

The Lowry method proved most satisfactory for this work because the protein content in the samples was so small, but the Biuret colorimetric reaction was also useful. The latter technique demonstrates that the cell walls contained from 10-20% protein, thereby supporting the results obtained from the Lowry method.

Qualitative analyses of the hydrolysed proteins from the cell walls disclosed the presence of 16 different amino acids. Listed in a descending order of concentration, the major amino acids in both samples were glutamic acid, aspartic acid, lysine and threonine (Table V). There were no apparent qualitative or quantitative differences in the amino acid complement of the 1 and 7 day samples, except perhaps for a slight increase of glutamic acid in the older cultures. Tryptophane and cystine residues may also be present in the cell walls, but were not detected because of technical limitations and the instability of these compounds during acid hydrolysis. It is important to note that the amino acid analysis of the cell wall proteins was only performed once. This decision was based on the fact that: (1) there is a good correlation of this data with the results previously obtained by Letourneau et al. (1976), (2) there does not appear to be a significant difference between the amino acid composition of the 1 and 7 day samples, and (3) the additional cost of further analyses.

(C) Carbohydrates

The class of compounds which maintain the structural integrity of the

TABLE V. Amino acid analyses of the proteins extracted from the cell walls of Choanephora cucurbitarum.

Amino Acid	% of Total Amino Acids	
	1 Day	7 Days
Lysine	8.7	8.6
Histidine	0.6	0.5
Arginine	3.6	4.0
Aspartic acid	14.1	13.4
Threonine	8.0	7.7
Serine	6.9	5.8
Glutamic acid	14.6	17.6
Proline	5.1	4.7
Glycine	4.9	5.2
Alanine	5.5	5.9
Valine	5.8	6.7
Methionine	0.6	0.5
Isoleucine	4.8	4.3
Leucine	6.9	6.9
Tyrosine	4.3	3.9
Phenylalanine	5.6	4.3

Note: Protein for this analysis was extracted with 5% TCA as described under Materials and Methods. The values presented are the result of 1 determination only.

cell wall are the carbohydrates. However, because they exhibit such a vast diversity of type and function, the sugars of the cell wall are difficult to qualitate and quantitate. Similarly, the upmost care must be taken to insure that the isolation techniques do not interfere with the native state of the carbohydrate, or erroneous data will result (Stradler, 1976). This investigation attempted to minimize these problems through the combined use of Spectrophotometric and GLC procedures.

The neutral sugars from the cell walls of C. cucurbitarum were extracted with a number of different acidic and basic reagents. The data reveals that the 7 day old sample contains significantly more neutral sugars than its 1 day old counterpart (Table III, Appendix I). After retreating the pellet with the respective solutions, the Anthrone test routinely detected the presence of an additional 0.5% neutral sugar by weight. These values are also included in the data presented in Table III.

The supernate from the 30% KOH treatment of the cell walls posed a unique problem. The high salt concentrations in these mixtures interferred with the various colorimetric assays, and attempts to neutralise or desalt the samples by dialysis proved futile. However, control experiments demonstrated that the alkali solution could be desalted with a Sephadex G-10 column, which had an 86% sugar recovery optimum (Appendix II). With this in mind, the results from the Anthrone test revealed that the 1 and 7 day old strong alkali soluble sugar fractions comprised 7.7% and 3.5% of the cell wall respectively (Table III). Furthermore, statistical analyses comparing the means of these samples verified that they were significantly different (Appendix I).

An interesting trend seems to have developed when the quantitative

data from the various sugar fractions are compared. When these values are totalled, the 1 and 7 day old cell walls of C. cucurbitarum contain approximately the same amount of sugars (ie) 28.1% and 29.9% of the cell wall dry weight respectively (Table III). This similarity, and the observation that the proportion sugar distribution in the young cell wall is opposite to that in the older cell wall, may indicate that there is a shift in the class of carbohydrates which is being synthesized during aging. Unfortunately, the data from this investigation cannot substantiate this hypothesis.

Subsequent hydrolysis and fractionation of carbohydrates extracted by acid and alkali treatments revealed that the neutral sugars fucose, mannose, galactose and glucose were found in both cell wall preparations (Table VI). Co-chromatography of a number of different sugar standards with the unidentified peak in the 1 day sample, failed to elicit a retention time which would have positively identified this compound. A similar problem was also encountered by Letourneau et al. (1976) in their cell wall studies. Only trace amounts of qualitatively similar neutral sugars were released upon re-hydrolysis of the residue left from the first treatment. This observation indicates that the hydrolysis of the carbohydrates was complete and without degradation, because no erroneous peaks appeared on the chromatograms.

Age appeared to have a dynamic effect on the neutral sugar complement of the cell wall fractions. From the 1 to 7 day incubation period, fucose and galactose decreased in abundance while mannose and glucose concentrations increased (Table VI, Figure 4). There is a significant difference between the mean concentrations of all the neutral sugars

TABLE VI The effect of age on the neutral sugar composition of the cell walls of *Choanephora cucurbitarum*

Incubation Period (days)	Neutral Sugars (% dry wt. cell wall)				
	Fucose	Mannose	Galactose	Glucose	Unknown
1	4.5 (0.31)	5.2 (0.07)	3.1 (1.16)	3.4 (0.4)	4.2 (0.38)
7	1.2 (0.11)	13.6 (0.14)	0.9 (0.03)	10.7 (0.01)	ND

Note: Figures in brackets refer to the standard deviation of the mean.

ND = not detected.

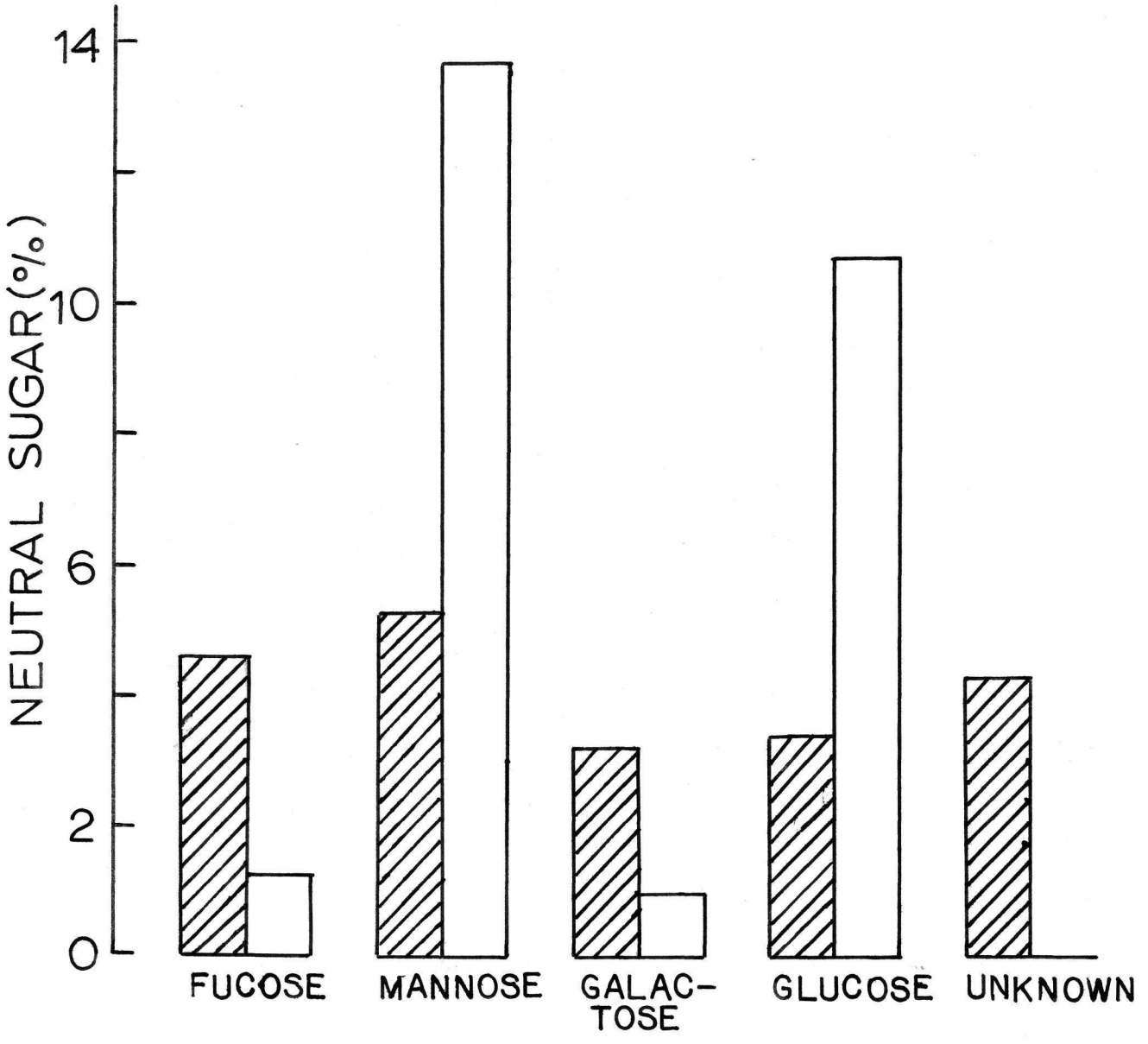


FIGURE 4 The effect of age on the neutral sugar composition of the cell walls of Choanephora cucurbitarum

▨ 1 day old cell walls

□ 7 day old cell walls

except galactose, when the 1 and 7 day old samples are compared (Table VI, Appendix I). The unequal variance values calculated from the galactose data prevented statistical analysis.

There were no amino sugar, uronic acids or amphoteric compounds detected in the supernates from the 1N NaOH, 1N acetic acid or 30% KOH treatments. This result was particularly surprising in the case of the amino sugar chitosan, because it is known to be soluble in acetic acid (Bartnicki-Garcia and Nickerson, 1962). However, these authors pretreated the cell walls with HCl prior to acetic acid extraction, and this may be the reason why chitosan was not detected in the present investigation. One important point which is evident from these studies is however, the apparent absence of unbound N-acetylglucosamine or glucosamine which would have otherwise appeared in the extracts. On the other hand, the lack of uronic acids in these fractions may be due to their decomposition during the hydrolysis procedure (Datema et al., 1977b). Finally, the fact that no amphoteric compounds were detected is postulated to be the result of the limitations of the gravimetric technique. The possibility also exists of course, that the cell wall does not contain any of these compounds.

The pellet which remained after these treatments was presumably composed of chitin, chitosan and perhaps uronic acid. Attempts to qualitate and quantitate these amino sugars using different hydrolysis techniques proved futile, because chitin was often deacetylated during the acid treatment (Siestma and Wessels, 1977). However, in the nitrous acid method this problem is solved. Only amino sugars without a protective acetyl group will be depolymerized, leaving chitin as the insoluble residue (Datema et al., 1977b). Therefore, careful hydrolysis and

quantification of the amino sugars in the nitrous acid treated and untreated cell wall samples yields their chitin and chitosan content. Using this technique, the data reveals that the 1 day old cell wall of C. cucurbitarum contains 16.8% and 18.4% of chitin and chitosan respectively (Table III). Conversely, the 7 day old sample was made up of significantly less chitosan and more chitin then its counterpart. By converting these figures into a chitin: chitosan ratio, it becomes apparent that the near unity value of the 1 day old cell wall, changes to a 2:1 ratio in the older hypha (Table III). The cell walls of the sporangiophore of M. rouxii exhibit a chitin: chitosan ratio which closely resembles the data presented for our 1 day sample. However, there is no correlation of our results with the chitin: chitosan ratios in evidence in the vegetative forms of M. rouxii (Bartnicki-Garcia and Reyes, 1964 and 1968; Bartnicki-Garcia and Nickerson, 1962).

The quantitative evaluation of the chitin content in the 2 day old cell walls of C. cucurbitarum by Letourneau et al. (1976) is in close agreement with that which was observed in the young hypha in the present study. However, it is important to point out that the chitosan content reported in this investigation is significantly lower then that which is published in the literature for other Mucoraceous species (Letourneau et al., 1976; Bartnicki-Garcia, 1968; Frey, 1950). We postulate that this discrepancy may have been caused by the hydrolysis of the amide bond and generation of deacetylated chitin in those reports (Siestma and Wessels, 1977). Finally, the total percentage of the cell wall which is made up of amino sugars in the 1 and 7 day old samples is 35.2% and 31.3% respectively (Table III). The total hexosamine content in the cell walls of Mucor mucedo closely resembles these figures (Datema et al., 1977b).

Infrared spectrophotometry and x-ray diffractometry revealed that the nitrous acid insoluble residue was chitin. The infrared spectrophotometric tracings from the authentic chitin standard and the cell wall chitin fraction showed peaks at 840, 900, 950, 1030, 1060, 1120, 1170, 1200, 1270, 1380, 1460, 1575, 1670, 1700 and 1710 cm^{-1} (Figure 5). In the second technique, the similarity of the interplanar spacings in these x-ray diffraction patterns confirmed that the nitrous acid insoluble fraction from the cell walls is chitin (Figure 6).

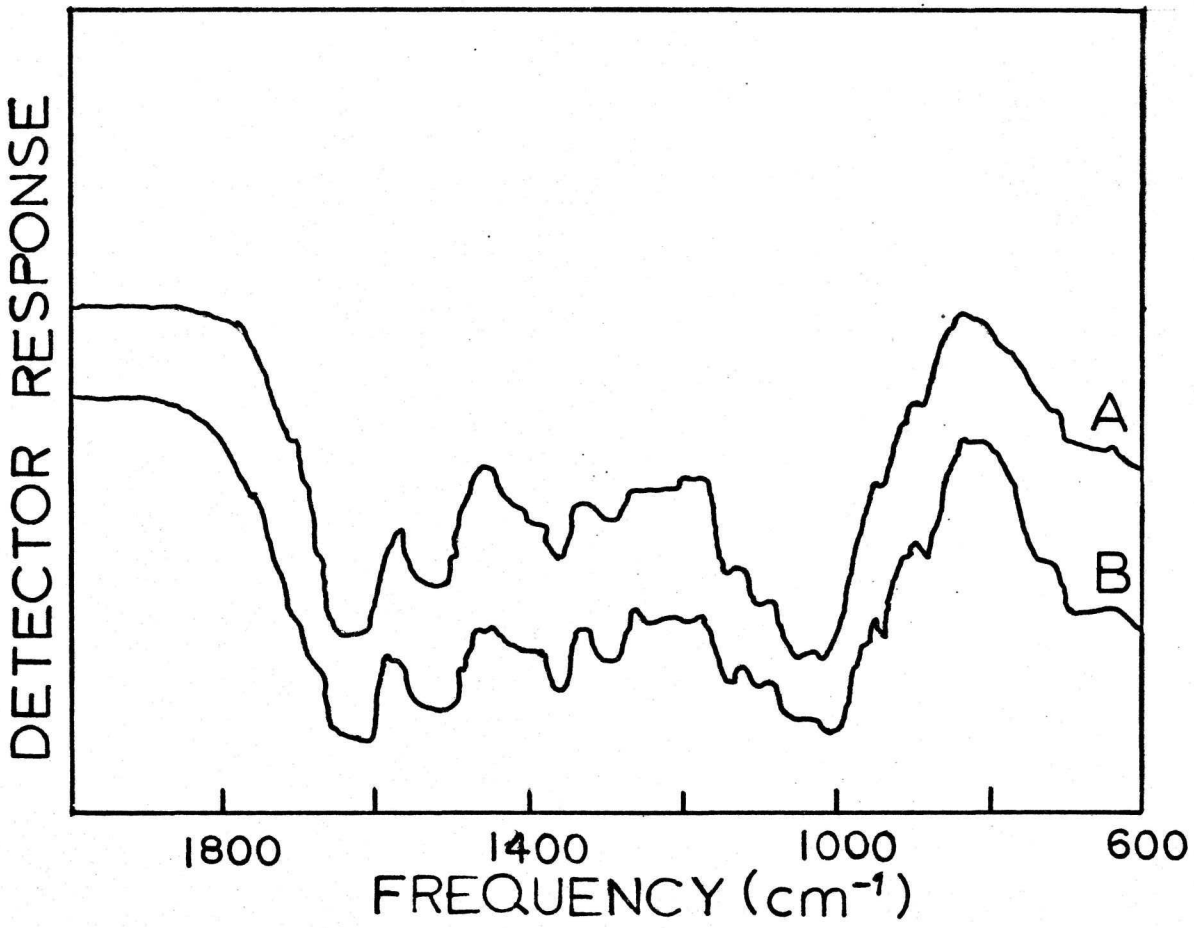
The uronic acid content of the 1 and 7 day old cell walls of C. cucurbitarum was approximately 1% of the total dry weight (Table III). Similarly, there was no significant difference between the quantitative values recorded for either of the incubation periods. The qualitative nature of the uronic acids was not analyzed, but earlier studies revealed the sole presence of glucuronic acid in these cell walls (Letourneau et al, 1976).

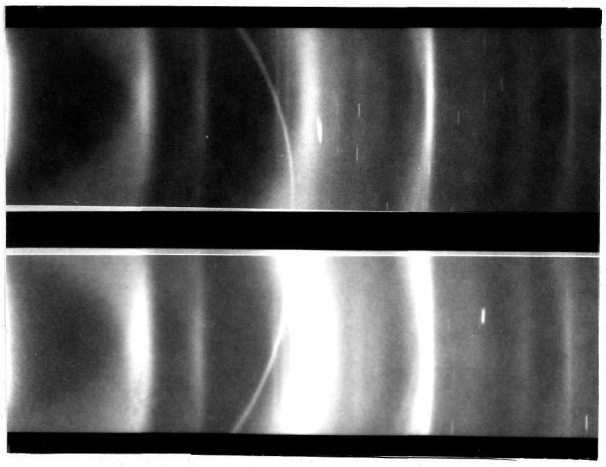
The calculated values of the standard deviations for each of the carbohydrate fractions rarely exceeded 1% of the total cell wall dry weight (Table III). These values were taken into consideration during the interpretation of the results, particularly in instances where the standard deviation appeared abnormally high (ie) neutral sugars, 7 day old cell wall sample (Table III). There is little doubt that the variability of the data is due to the experimental error which is applicable to any technique. This point is illustrated by the relative proportionality of the sample standard deviation with the actual experimental values (Table III).

(D) Ash Content

The ash content of the cell walls of C. cucurbitarum was determined

Figure 5 Infared spectrophotometry and x-ray diffractometry analyses of authentic (A) and purified cell wall chitin (B)


DETECTOR RESPONSE
A
B
1800
1400
1000
600
FREQUENCY (cm⁻¹)


A
B

to have an average value of 4.8% of the dry weight. There was a slight but insignificant decrease in the ash content as the cultures aged (Table III). The qualitative nature of this fraction was not examined in this investigation.

III Structure of the Cell Wall

In the previous section, it has become obvious that a dynamic change in the cell wall constituents is occurring over time. Since compositional changes are often expressed through and accompanied by structural variations, an attempt to verify this concept was made through the use of the electron microscope. Thin sections of 1 day old hypha revealed the presence of a homogenous, thin and smooth cell wall surrounding a complex cytoplasm (Figure 6). Adjacent to the cell wall is a closely appressed continuous structure which is representative of the plasmalemma. On the other hand, the cytoplasm of the cell is filled with a complex array of mitochondria, ribosomes and numerous types of vacuoles (Figure 6).

The older glutaraldehydeosmium fixed hypha possessed a differentiated cell wall (Figure 7). Two regions of different electron density appear within the 7 day old cell wall and signifies that depositor activity has occurred as this microorganism aged. The general morphology of the interior of the older cell was akin to that which was evident in its younger counterpart. The conspicuous presence of mitochondria, ribosomes and vacuoles illustrates this point (Figure 7).

The observation that the older cell wall was composed of two distinct regions confirmed earlier findings and implied that there was in fact, a definite link between structure and composition (Letourneau et al.,1976;

Figure 6 Thin section of 1 day old intact hypha of Choanephora cucurbitarum (x60,000). Note the presence of a monolayered cell wall.

Figure 7 Thin section of the 7 day old sample (x60,000). Note the appearance of a secondary layer.

6

7

Manocha and Lee, 1971). In order to correlate the different electron densities of the regions with possible structural changes, heavy metal replicas of the cell walls were prepared. The untreated cell wall was therefore coated with a Palladium-Gold alloy which enhanced the topography of the outermost layer. The amorphous or granular appearance of the cell walls from the 1 and 7 day old samples is illustrated in Figures 8 and 9 respectively. The amorphous nature of this region is apparently attributable to the chitosan, lipid and protein macromolecular complexes. Specific enzymatic removal of these molecules from the cell walls of other fungi is accompanied by the disappearance of their amorphous texture (Hunsley and Burnett, 1970).

Treatment of the samples with 1N NaOH for various times and at different temperatures failed to completely remove the amorphous components. However, hydrolysis of the cell walls in 1N HCl ($100^{o}C$, 1 hr.) was successful in stripping off the amorphous material and revealing a complex network of randomly oriented microfibrils. Figures 10 and 11 illustrate that this layer is common in both the 1 and 7 day old samples. In general, the microfibrils were tightly interwoven and had a diameter between 100 and 200 Å. Furthermore, the intersticies between the fibrils appeared to be filled with amorphous material.

Repeated hydrolysis of the 1 day old cell walls of C. cucurbitarum did not reveal any further changes in the microfibrillar structure. However, within the inner region of the older hypha there appeared a layer of parallel oriented microfibrils (Figure 12). Unfortunately, the orientation of these fibrils could not be elucidated since the extensive hydrolysis techniques had degraded the mycelial fragments into unrecognizable

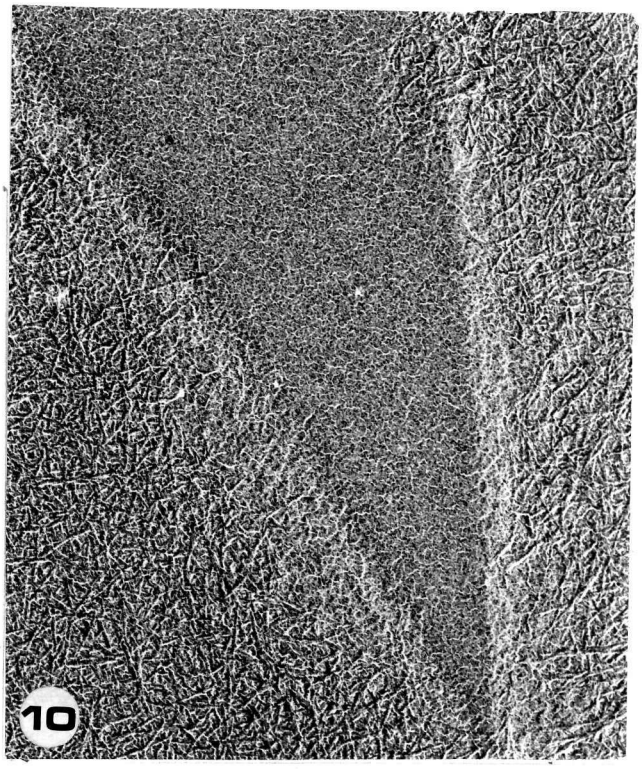
Figure 8 Heavy metal replica of the outer surface of the 1 day old cell wall of Choanephora cucurbitarum (x23,000).

Figure 9 Replica of the outer surface of the untreated 7 day old sample (x23,000). Note the similarity of the amorphous textures in both the young and old hyphae.

Figure 10 Replica of the acid treated 1 day old cell wall of Choanephora cucurbitarum revealing a network of random oriented microfibrils (x48,500).

Figure 11 Replica of a similarly treated cell wall fragment isolated from the 7 day old cultures (x48,500).


8


10


9

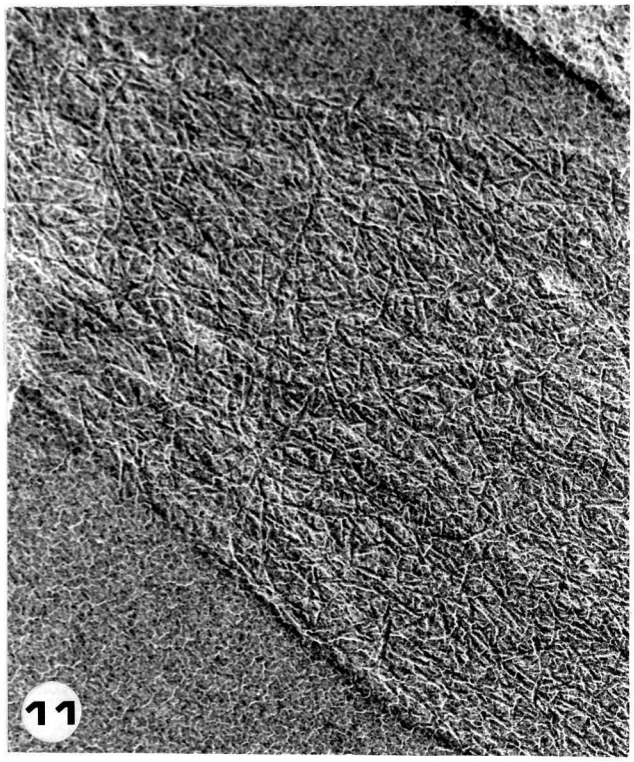
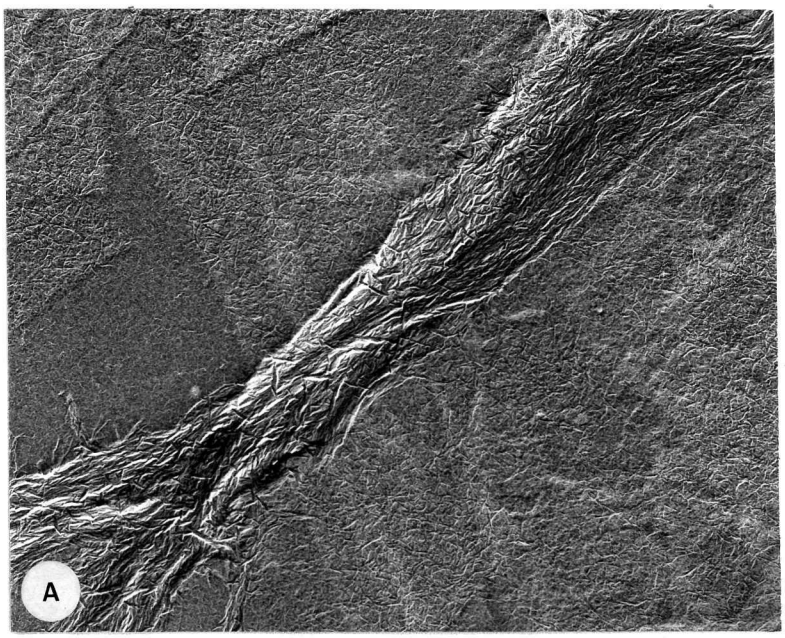
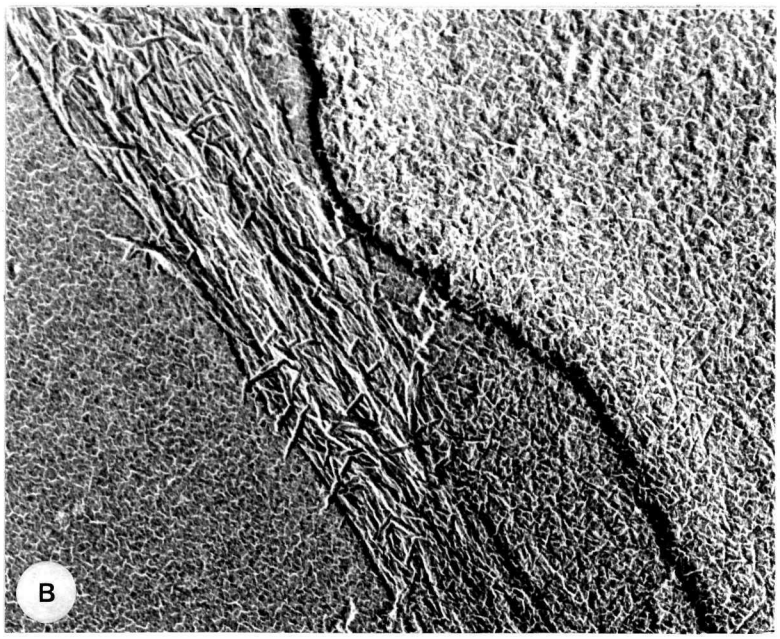

11

Figure 12(a) Replica of extensively hydrolyzed 7 day old cell walls of Choanephora cucurbitarum (x24,000). Note the presence of parallel oriented microfibrils.

Figure 12(b) Same as above but illustrated at a greater magnification (x48,500).


A


B

shapes. Overall though, except for the ordered parallel arrangements of this layer, the microfibril characteristics of this and the outer region were identical.

DISCUSSION

The cell wall is an important constituent of the fungal cell, since it acts as an effective barrier against environmental stress and yet is dynamic enough to be able to meet the demands of growth, development and morphogenesis (Rosenberger, 1976). There is little doubt that there is an innate link between the compositional and structural alterations which occur during these processes. However, investigators have found it impossible to interpolate any general relationship which exists between these two parameters, thereby creating a void in this aspect of fungal physiology (Bartnicki-Garcia and Reyes, 1968).

To our knowledge, there are very few publications which present a comprehensive viewpoint relating the effect of age on the structure and composition of the fungal cell wall. The completion of this investigation has therefore added a new dimension to a developing field. In order that a clear interpretation of the results from this study can be presented, the following discussion is divided into two subsections.

I Composition of the Cell Wall

Cell wall fractions from cultures of Choanephora cucurbitarum which were confirmed to be free of cytoplasmic contamination, routinely made up from 17.6% to 17.9% of the total mycelial dry weight. In general, cell wall yield is a species specific phenomena. This point is illustrated by the values reported for the filamentous hyphae of Mucor rouxii and Paracoccidiodes brasiliensis, whose cells contain 14.2% and 8% dry weight cell wall (Moreno, 1969; Bartnicki-Garcia and Nickerson, 1962). The variation in the cell wall yield which is apparent in the literature however, may be due to the purity of the sample following isolation or to the

method which was used for its enumeration. In the latter case for example, variations in the daily laboratory conditions or incomplete dessication of the cell wall samples will undoubtably influence the gravimetric data. In the present investigation, the less then 0.5% deviation of the experimental values about the mean is an acceptable level of error considering the limitations of the technique.

There does not appear to be a significant effect of age on the amount of cell wall in the 1 and 7 day old hyphae of C. cucurbitarum. This result was not expected since the older cell wall is known to undergo the deposition of a secondary layer, and which would presumably be accompanied by a greater weight. However, changes in the orientation, composition or degree of interaction of the cell wall elements may occur during aging and therefore affect the appearance of the cell wall under the electron microscope (Rosenberger, 1976). Consequently, this implies that the apparent thickness of the cell wall may not be directly proportional to its actual weight. This point is reinforced by earlier findings of Bartnicki-Garcia and Nickerson (1962) on the dimorphic forms of M. rouxii. The yeast cell wall appeared 10 times thicker then its filamentous counterpart, but in turn contained only 25% more cell wall material. We therefore postulate that the secondary layer in the older hypha of C. cucurbitarum does not add enough weight to the cell wall in order for it to be reflected in the gravimetric data.

The 1 and 7 day old cell walls of C. cucurbitarum characteristically contained lipid, protein, neutral sugars, chitin, chitosan and uronic acids. (Table III). In order to confirm the accuracy of the quantitative data presented in this investigation, each of these classes of compounds were extracted by several different techniques. On the other hand,

qualitative identification of the constituent compounds was made through the use of authentic standards.

The data reveals that the cell wall of C. cucurbitarum contains lipids, and therefore lends support to the contention that lipids are not a cytoplasmic contaminate, but rather are an integral part of this structure (Dyke, 1964). Although the precise role of lipids in the cell wall is not entirely clear, some investigators have inferred that these compounds are intimately associated with structural, enzymatic and physiological processes which occur within the cell wall (McMurrough et al., 1971; Nickerson, 1963; Kreger, 1954). However, the results from the present investigation were unable to ascertain the role of the lipid material in the cell wall of this fungus.

The total amount of lipid that was extracted from the 1 and 7 day old cell walls of C. cucurbitarum is 6.1% and 8.1% of the dry weight respectively. These values are not in agreement with those which were reported by Letourneau et al. (1976), who were able to isolate approximately twice as much lipid material from their cell wall samples. This discrepancy may be due to the inherent error of the gravimetric technique, or to the differences in the ages of the cultures from which the cell walls were isolated. In general however, it is important to note that the lipid content of the cell walls from other Mucoraceous fungi studied thus far rarely exceeds 5% of the dry weight (Bartnicki-Garcia and Reyes, 1964 and 1968; Bartnicki-Garcia and Nickerson, 1962).

The data indicate that there is a slight increase in the lipid content of the cell walls as they age. The significance of this change could not be evaluated statistically because the calculated standard deviations of the mean values were not equal. To our knowledge, there are no published

accounts which outline the effect of age on the lipid composition of a fungal cell wall. However, fluctuations in the lipid content of the total fungal cell during the aging phenomenon is routinely observed in other investigations (Devan and Manocha, 1976).

During the methylation and hydrolysis of the cell wall lipids, the volitile fatty acid methyl esters are cleaved from their polar head groups and analysed by Gas-Liquid Chromatography (GLC). The qualitative presence of lauric, myristic, palmitic, palmitoleic, stearic and oleic acids in both the 1 and 7 day old samples in the present investigation is confirmed by the earlier work of Letourneau et al. (1976) (Table IV). Similarly, the presence of linoleic and γ-linolenic acid in the older cell wall was also observed in the 2 day old cell wall fractions from this same study (Letourneau et al., 1976). It appears that these long chained fatty acids are being incorporated into the cell wall during the earlier stages of growth. However, no explanation for this physiological response can be given at this time, since relative data in this area is very limited.

The degree of unsaturation of the fatty acids increases significantly as the cell wall ages (Table IV). There is little doubt that this observation is directly correlated to the concomitant increase in the concentrtion of the long chained unsaturated fatty acids. Utilizing cultural parameters which would increase the concentration of γ-linolenic in the cell fractions, Devan and Manocha (1976) were also able to correlate an increase in the degree of unsaturation of the total fatty acid composition of C. cucurbitarum with its γ-linolenic acid content. Alternately, in Nadsonia elongata, Dyke (1964) observed that the fatty acids of the whole hyphae were mainly unsaturated whereas those in the cell wall were generally saturated in nature. This trend is also evident in the present

study, particularly in the instances where the cultural conditions for both samples are closely matched (Devan and Manocha 1976).

Cell wall proteins are postulated to play a major role in the formation of macromolecular complexes within this structure (Rosenberger, 1976; Crook and Johnston, 1962). The present investigation revealed that as much as 17% of the dry weight cell wall was protein (Table III). However, its native state within the cell wall of C. cucurbitarum was beyond the scope of this study. Cell wall protein concentrations within the various fungal groups is both a species and structure specific phenomena. For example, in Fusarium spp. the cell wall protein concentration of the vegetative and reproductive forms is 7.3% and 21% of the dry weight respectively (Barran et al., 1975). This type of variability is also characteristic of the fungi in the order Mucorales. In general however, the protein concentrations observed in the present study were greater than that which had been reported previously (Letourneau et al., 1976; Bartnicki-Garcia and Reyes, 1964 and 1968; Bartnicki-Garcia and Nickerson, 1962). No explanation for this finding can be given, but it is important to point out that various methods of extracting and quantitating the protein content were used in order to verify these values.

There is a slight but significant increase in the polypeptide content in the cell walls as the cultures aged. A similar trend is also observed during secondary cell wall deposition in cotton fibres (Huwyler et al., 1979) and the aging of Saccharomyces cerevisiae cells (McMurrough and Rose, 1967).

The characteristic acidic nature of the amino acids commonly reported in fungal cell walls is also apparent in this study (Rosenberger, 1976; Nickerson, 1963). This point is illustrated by the fact that approxim-

ately 30% of the total amino acid complement is made up of aspartic and glutamic acids (Table V). There is a very good correlation of the qualitative and quantitative nature of the amino acid composition detected in this investigation with that which was reported by Letourneau et al. (1976). The conspicuous absence of hydroxyproline in the chitinous walls of C. cucurbitarum, further reinforces the contention that this amino acid is restricted to microorganisms which contain cellulose in their cell walls (Crook and Johnston, 1962). Some authors postulate that the presence of cysteine residues in the protein fraction is indicative of disulphide bridge formation between the cell wall constituents (Barran et al., 1975). However, the limitations of the amino acid analysis technique which was used in the present investigation precludes further comment, as cysteine residues are unstable in vitro.

There are essentially no significant differences illustrated between the 1 and 7 day old amino acid fractions (Table V). Similarly, the slight increase in the glutamic acid content of the protein over the incubation period is postulated to be within the limits of experimental error and therefore considered insignificant.

The total neutral sugar concentration in the 1 and 7 day old cell wall samples of C. cucurbitarum was higher than that which has been previously reported (Letourneau et al., 1976). Normally, under identical hydrolyses conditions the Mucoraceous cell wall contains from 5% to 10% of the dry weight neutral sugar (Bartnicki-Garcia and Reyes, 1964 and 1968; Bartnicki-Garcia and Nickerson, 1962). However, when these conditions are varied and the appropriate precautions have been taken, there is often a substantial increase in the neutral sugar yield. In particular, the disappearance of the pentoses and deoxyhexoses in Mucor mucedo cell walls

was attributed to be the result of extensive and destructive hydrolysis techniques (Weijman and Meuzelaar, 1979). Similarly, careful treatment of Phycomyces blakesleeanus cell wall fractions resulted in a 30% yield of the dry weight of the neutral sugars (Van Laere et al., 1977). There is no doubt that the carbohydrate concentration is a species specific phenomena. However, it is important to point out that these differences may also be magnified by experimental technique. We therefore postulate that these factors must also be considered when the data from the present investigation is compared to that of the literature.

There is a significant increase in the total neutral sugar fraction, and a concurrent and proportional decrease in the strong alkali soluble sugar fraction as the cultures aged (Table III). The net effect of these changes however, resulted in a nearly equal total carbohydrate concentration in the 1 and 7 day old cell walls of C. cucurbitarum. It appears from these observations that a shift in the class of carbohydrates which are being incorporated into the cell wall is occurring as the fungal hypha ages. Similar quantitative changes in the different carbohydrate fractions of aging barley and cotton cell walls has also been reported (Huwyler et al., 1979; Morrall and Briggs, 1978). These authors were unable to elucidate any general trends which would logically relate cell wall development to its carbohydrate constituents. A similar problem exists in this area of fungal physiology until sufficient data is available. It is also important to point out that the solubility of a carbohydrate moiety is directly related to its accessibility by the particular solvent. The dynamic nature of the cell wall components during growth could conceivably mask the effect of solvents on otherwise reactive groups, and therefore account for the changes which were observed in the present study (Storck and Price, 1977).

Identification of the neutral sugar constituents in the cell walls of C. cucurbitarum GLC revealed the presence of fucose, mannose, galactose and glucose (Tabel VI). This finding is in general agreement with the characteristic sugar complement of fungi from the order Mucorales with one exception (Letourneau et al., 1976; Bartnicki-Garcia and Reyes, 1968; Bartnicki-Garcia and Nickerson, 1962). Glucose is not normally considered to be a constituent of the vegetative cell wall although it is the dominant carbohydrate in the Mucoraceous spore cell wall (Bartnicki-Garcia and Reyes, 1964). However, recent investigations by Van Laere et al. (1977) and Weijman and Meuzelaar (1979) supports this study since these authors have also reported the presence of glucose in the hyphal wall of P. blakesleeanus and M. mucedo respectively. It therefore appears that the contention of Bartnicki-Garcia (1968) that the absence of this carbohydrate in the vegetative cell wall of Zygomycetes is a distinguishing feature of this group, must be reconsidered. It is becoming exceedingly clear that the cultural parameters play a significant role in determining the absolute composition of the cell wall, particularly with respect to the minor constituents.

The nearly equal distribution of the neutral sugars in the 1 day old cell wall sample is analogous to that which was observed in M. mucedo and Allomyces ssp. (Weijman and Meuzelaar, 1979). On the other hand, as the hypha aged the individual neutral sugar changed in abundance. Similar quantitative alterations in the monosaccharide content of different structures or dimorphic forms of M. rouxii have also been observed (Bartnicki-Garcia and Reyes, 1964 and 1968; Bartnicki-Garcia and Nickerson, 1962). We can offer no explanation for the concurrent decrease of fucose and galactose, and increase of mannose and glucose as the cultures age

(Figure 4). However, it does seem that the latter two monosaccharides are associated with secondary cell wall formation in C. cucurbitarum, since they are in greater abundance in the older fractions.

The major building blocks in the cell walls of C. cucurbitarum are the amino sugars, chitin and chitosan. The qualitative presence of these hexosamines follows and substantiates the phylogenetic relationship of this fungi with other Zygomycetes, as postulated by Bartnicki-Garcia (1968) (Table I). Since chitin is unequivocally involved in the formation of microfibrils, its role in maintaining the structual integrity of the cell wall is self-evident (Rosenberger, 1976). The ability of chitosan to form similar networks is presently unclear, and therefore chitosan is generally regarded to be an integral part of the matrix of the cell wall (Storck and Price, 1977).

Approximately 33% of the cell wall of C. cucurbitarum is composed of chitin and chitosan. This value is in agreement with the total hexosamine concentration in the cell walls of the yeast form of M. rouxii (Bartnicki-Garcia and Nickerson, 1962). Similarly, the proportion of the total amino sugar fraction which has been identified as being chitin in the present study, is analogous to that which had been reported previously by Letourneau et al. (1976). However, an inconsistency develops when the chitosan concentrations between these two studies are compared. Letourneau et al. (1976) reports that the two day old cell walls of C. cucurbitarum contain 28% of the dry weight. This value does not correlate with our data, but this may be due to the fact that the samples were taken at different incubation periods. On the other hand however, we postulate that the descrepancy could be linked to the extraction technique which was used by these workers. In their study, the hydrolysis of the cell

wall sample with 6N HCl (105°C, 12 hr.) would inevitably release and deacetylate the N-acetylglucosamine residues from the parent chitin molecule and thereby appear as glucosamine in the GLC chromatograms (Siestma and Wessels, 1977; Letourneau et al., 1976). This problem was overcome in the present study because the nitrous acid will only hydrolyse the amino sugars without a protective acetyl group (Datema et al., 1977b).

The most significant change in the cell wall of C. cucurbitarum as it ages is the shift in the chitin: chitosan ratio. The near unity of this value in the younger cultures changes significantly to a 2:1 ratio in the 7 day old samples (Table III). The sporangiophore cell wall of M. rouxii also contains equal amounts of chitin and chitosan, and therefore resembles the 1 day old hypha. Alternately, the increase in the chitin content in the older cell walls of C. cucurbitarum which was observed in the present investigation is also evident in Allomyces spp. (Aronson and Machlis, 1975). Given that the amino sugar component of the cell wall is a major contributor to the skeletal basis of this structure, we contend that the properties of the cell wall of this fungus have changed during the aging phenomenon. Similarly, since chitin is the major component of microfibrils then we can postulate that the noted increase in the chitin content in the 7 day old cell walls is accompanied by an increase in the rigidity of this structure (Hunsley and Burnett, 1970).

The uronic acid component of the cell wall of C. cucurbitarum does not appear to play a significant role in its structure or composition (Table III). This fact is generally consistent within the vegetative hyphae of Mucorales, although some exceptions do exist. The sporangiophore of M. rouxii are known to contain up to 25% of their dry weight of

glucuronic acid (Letourneau et al. 1976; Bartnicki-Garcia and Reyes, 1964 and 1968; Bartnicki-Garcia and Nickerson, 1962). Thus far, the appearance of uronic acids in cell walls has been reported in a number of different classes of fungi which are essentially unrelated (Barran et al. 1975; Crook and Johnson, 1962). However the seemingly limited distribution of these compounds may be due to the fact that researchers have unknowingly destroyed the uronic acids in their samples through treatment with strong mineral acids (Rosenberger, 1976; Gancedo et al., 1966). Special consideration was given to this point in the present study.

There is little doubt that the ash content of the cell wall is both a species and structure specific phenomenon (Bartnicki-Garcia, 1968). This variability within the literature is compounded further by the inherent variations within the drying and gravimetric techniques which are used to establish the ash content of the cell wall. We believe that these factors may be responsible for the 10% increase in the ash composition of the cell walls of C. cucurbitarum which was previously reported by Letourneau et al. (1976), in comparison to that which was found in the present investigation.

II Structure of the Cell Wall

It has become quite evident thus far that age has a dynamic effect upon the composition of the cell wall, particularly with respect to its major components. Since each constituent imparts a different degree of stainability to the cell wall, the deposition of a number of like molecules in the same region will result in the appearance of a layer. Similarly, microfibril forming compounds such as chitin will give the cell wall a

definable topographical morphology which is discernable through the auspicious use of heavy metal shadow casing techniques (Rosenberger, 1976). Since there is a specific need for correlating the compositional and structural changes in the cell wall as it ages, the following discussion will outline any trends which have developed and can serve as a basis for further investigations.

The young coenocytic hypha of C. cucurbitarum was observed to possess a monolayered cell wall of uniform electron density (Figure 6). The appearance of the 1 day old cell wall in this study is in accord to that which was observed by Letourneau et al. (1976) and Manocha and Lee (1971). The filamentous vegetative form of M. rouxii also exhibited the same type of cell wall construction. However, this point is not surpiring in light of the fact that these cultures were only 12 hr. old (Bartnicki-Garcia and Nickerson, 1962).

Our data confirm the previous reports that the older hypha of C. cucurbitarum develops a secondary cell wall (Letourneau et al., 1976; Manocha and Lee, 1971). It is interesting to note that the 4 day old cell walls of the yeast form of M. rouxii and the filamentous hypha of Neurospora crassa also possess a bilayered cell wall, but it is not known whether this is the result of the phenomenon of secondary cell wall deposition (Mahadevan and Tatum, 1967; Manocha and Colvin, 1967; Bartnicki-Garcia and Nickerson, 1962). It has been postulated that the layering which is apparent in some fungal cell walls is due to the inability of the cell to incorporate new material into the existing structure (Hunsley and Burnett, 1970). The increase in the protein, neutral sugar and chitin content in the cell wall of C. cucurbitarum as it ages is correlated with the formation of a secondary layer (Table III, Figure 7). The above hypothesis

thereby implies that this material is part of the secondary layer. However, we can only speculate that this trend is being followed in the present investigation. Further studies involving the specific hydrolysis of molecules from an intact cell wall fragment are required before this prediction can be substantiated.

The Pd-Au alloy replicas from the untreated 1 and 7 day old cell wall samples had a relatively uniform, finely granular appearance (Figures 8 and 9). In general, the outermost surface of the fungal cell wall is characteristically described as being devoid of any ordered array of structures, but rather is composed of amorphous components (Barran et al., 1975; Hunsley and Burnett, 1970; Bartnicki-Garcia and Lippman, 1969). The amorphous components are the proteins, lipids and chitosan of the cell wall, which are unable to form macromolecular complexes which are distinguishable by the heavy metal replica technique. Consequently, the compositional changes which were occurring as the cell wall aged are not reflected by the topography of the outer cell surface. On the other hand however, the apparent similarity of the 1 and 7 day old cell wall surfaces supports the contention that the cell is unable to incorporate new material into the already existing structure (Hunsley and Burnett, 1970).

Acid hydrolysis of the purified cell wall samples revealed the presence of a microfibrillar chitin net which was embedded within, and in close association to the amorphous constituents of the outer layer (Figures 10 and 11). The appearance of the random oriented microfibrils in both the 1 and 7 day old cell wall samples in the present investigation was in accord with that which was observed previously (Letourneau et al., 1976; Manocha and Lee, 1971). Similarly, the outer layer of the cell walls of

P. brasiliensis, Pythium debaryanum and N. crassa also demonstrate this type of fibril configuration (Moreno, 1969; Manocha and Colvin, 1967 and 1968). It is interesting to note that the density, orientation or thickness of the microfibril component does not vary between the 1 and 7 day old cell wall samples. This observation is analogous to that which was outlined by Bartnicki-Garcia and Nickerson (1962) in the dimorphic forms of M. rouxii. In particular, despite the enormous differences in the shape and thickness of the cell walls of the yeast and filamentous forms, there is a continuity in the structural elements within the microfibril mesh. On the other hand however, one must consider that the viability of the cell is dependent on the ability of the cell wall components to reorganize and compensate for the demands of growth (Rosenberger, 1976). Therefore, the possibility that these events are occurring within the cell walls of C. cucurbitarum does exist, but remained undetected due to the resolution of the technique.

Further treatment of the 1 day old cell wall with hot HCl confirmed that this structure was in fact monolayered and composed solely of random configurations of microfibrils. Conversely, the older hypha demonstrated that the second region was composed of a layer of parallel oriented microfibrils (Figure 12). These observations confirmed the earlier findings of Letourneau et al. (1976) in the older cell walls of C. cucurbitarum, and were analogous to the structure of P. debaryanum cell walls (Manocha and Colvin, 1968). It is difficult to explain the deposition of a parallel microfibrillar layer with respect to the mechanisms which are believed to be responsible for their formation. The current hypothesis predicts that the cytoplasmic microtubules control the orientation of the cell wall microfibrils. Disruption of the cytoplasmic skeleton often results in the

deposition of non-aligned microfibrils in some fungi (Marchant and Hines, 1979; Ruiz-Herrera et al., 1975; Colvin, 1972). When the cells of C. cucurbitarum age therefore, it follows that its cytoplasmic microtubules re-oriented before the secondary cell wall was deposited. Unfortunately, the scope of the present study was not within the limits required to evaluate this problem.

The correlation between the increase in the concentration of N-acetyl-glucosamine and the formation of a secondary cell wall, demonstrates the important role that chitin assumes in the aging process. When this factor is coupled with the high degree of resistance to acid hydrolysis which the secondary layer exhibits, then there can be little doubt that the parallel oriented microfibrils are composed of chitin. In future investigations, the judicious use of specific enzymes which will preferentially hydrolyse the parallel-oriented microfibrils will support this hypothesis.

The importance of the data presented in this report is not only reflected in the physiology of the aging process, but also in the role of the cell wall in the host: parasite relationship. In the mycoparasitic system involving the host: C. cucurbitarum, and the parasite: Piptocephalis virginiana, the host becomes resistant to infection as the hypha ages (Manocha and Golesorki, 1979; Manocha and Lee, 1971 and 1972). Since the cell wall is the first barrier which P. virginiana must circumvent in order to derive the necessary nutrients from the host, it is the structure or composition of its various layers which will determine the success or failure of the infection (Brian, 1976). We therefore speculate that the parasite is unable to establish a successful nutritional relationship with the older host because of (1) the change in the chitin: chitosan ration in its cell wall, and (2) the formation of a secondary layer. In order to

substantiate this hypothesis however, one must carry out experiments which would involve infecting mutant strains of C. cucurbitarum which are unable to develop a secondary cell wall. Alternately, inhibition of chitin synthesis in growing host cultures by Polyoxin-D would essentially create a similar net effect. The successful parasitism by P. virginiana of these hosts would thereby verify the role of the secondary cell wall in the myco-parasitic system.

Upon completion of this investigation, it has become increasingly clear that further work on this aspect of fungal physiology is needed at this time. Under specific and standardized cultural conditions, further analyses of the cell wall will enable the experimentor to build on the trends which are established in this investigation. The eventual accumulation of this type of data will therefore lead to the elucidation of the composition: structure relationship. This study has laid the groundwork for further experiments which should entail the specific labelling of the cell wall components followed by electron or light microscopic observation. Alternately, the removal of these constituents through the auspicious use of enzymes will serve towards localizing their position in the cell wall. Perhaps however, the most important point which has been brought to light in this investigation is the inherent plasticity of the cell wall. Certainly, the classical concept of the cell wall as being an inert structural entity has no basis in modern science.

LITERATURE CITED

Aronson, J. M., and L. Machlis. 1959. The chemical composition of the hyphal walls of the fungus Allomyces. Amer. J. Bot. 46 : 292-300.

Asamizu, T., and A. Nishi. 1979. Biosynthesis of the cell wall polysaccharides in cultured carrot cells. Planta, 146 : 49-54.

Barnett, H. L., and V. Lilly. 1950. Effect of nutritional and environmental factors upon asexual reproduction of Choanephora cucurbitarum in culture. Phytopathology, 14 : 80-89.

Barran, L. R., Schneider, E. F., Wood, P. J., Madhosingh, C., and R. W. Miller. 1975. Cell wall of Fusarium sulphureum. (I) Chemical composition of the hyphal wall. Biochim. Biophys. Acta, 392 : 148-158.

Bartnicki-Garcia, S. 1963. Symposium on biochemical bases of morphogenesis in fungi. (III) Mold - yeast dimorphism of Mucor. Bacteriol. Rev. 27 : 293-304.

Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. 22 : 87-108.

Bartnicki-Garcia, S., and E. Lippman. 1969. Fungal morphogenesis : Cell wall construction in Mucor rouxii. Science, 165 : 302-303.

Bartnicki-Garcia, S., Nelson, N., and E. Cota-Robles. 1968. Electron microscopy of spore germination and cell wall formation in Mucor rouxii. Arch. Mikrobiol. 63 : 242-255.

Bartnicki-Garcia, S., and W. J. Nickerson. 1962. Isolation, composition and structure of cell walls of filamentous and yeast-like forms of Mucor rouxii. Biochim. Biophys. Acta, 58 : 102-119.

Bartnicki-Garcia, S., and E. Reyes. 1964. Chemistry of spore wall differentiation in Mucor rouxii. Arch. Biochem. Biophys. 108 : 125-133.

Bartnicki-Garcia, S., and E. Reyes. 1968. Chemical composition of sporangiophore walls of Mucor rouxii. Biochim. Biophys. Acta, 165 : 32-42.

Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. 4 : 330-334.

Bligh, E., and W. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37 : 911-917.

Blumenthal, H. J., and S. Roseman. 1957. Quantitative estimation of chitin in fungi. J. Bacteriol. 74 : 222-225.

Bracker, C. E. 1966. Ultrastructural aspects of sporangiospore formation in Gibertella persicaria. In : The fungus spore. Edited by M. F. Madelin. Butterworth Press. London. pp. 39-60.

Bracker, C. E., and N. K. Halderson. 1971. Wall fibrils in germinating sporangiospores of Gibertella persicaria. Arch. Mikrobiol. 77 : 366-371.

Bracker, C. E., and L. J. Littlefield, 1971. Structural concepts of host-pathogen interfaces. In : Fungal pathogenicity and the plants response. Edited by R. J. W. Byrde and C. V. Cuthing. Academic Press. N. Y. pp. 159-317.

Brian, P. W. 1976. The phenomenon of specificity in plant disease. In : Specificity in plant disease. Edited by R. K. Wood and A. Graniti. Plenum Press. N. Y. pp. 15-26.

Burnett, J. H. 1970. Fundamentals of mycology. Edward Arnold Publishers Ltd. London. pp. 17-59.

Colvin, J. R. 1972. The structure and biosynthesis of cellulose. Crit. Rev. Macromol. Sci. 1 : 47-80.

Crook, E. M., and I. R. Johnston. 1962. The qualitative analysis of the cell walls of selected species of fungi. Biochem. J. 83 : 325-331

Datema, R., Van Der Ende, H., and J. G. H. Wessels. 1977(a). The hyphal wall of Mucor mucedo. (1) Polyanionic polymers. Eur. J. Biochem. 80 : 611-619.

Datema, R., Wessels, J. G. H., and H. Van Der Ende. 1977 (b). The hyphal wall of Mucor mucedo. (2) Hexosamine containing polymers. Eur. J. Biochem. 80 : 621-626.

Dawson, R. M. C., Elliot, D., Elliot, W. H., and K. M. Jones (editors). 1969. Data for biochemical research (second edition). Oxford University Press. London.

Deven, J. M., and M. S. Manocha. 1976. Effect of various culture conditions on the fatty acid and lipid composition of Choanephora cucurbitarum. Can. J. Microbiol. 22 : 443-449.

Dyke, K. G. H. 1964. The chemical composition of the cell wall of Nadsonia elongata. Biochim. Biophys. Acta, 82 : 374-384.

Folch, J., Lees, M., and G. Sloane-Stanley. 1957. A simple method for isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226 : 497-509.

Frey, R. 1950. Chitin and zellulose in Pilzzelwanden. Ber. Schweiz. Botan. Ges. 60 : 199-230.

Fultz, S. A., and A. S. Sussman. 1966. Antigenic differences in the surface of hyphae and rhizoids in Allomyces. Science, 152 : 785-787

Gancedo, J. M., Gancedo, C., and C. Asensio. 1966. Uronic acids in fungal cell walls. Biochemische Zeitschrift, 346 : 328-332.

Ghuysen, J. M. 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bacteriol. Rev. 32 : 425-464.

Gornal, A. G., Allan, G., Bardawill, C. J., and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177 : 751-761.

Hawker, L. E. 1966. Germination : morphological and anatomical changes. In : The fungus spore. Edited by M. F. Madelin. Butterworth Press. London. pp. 151-163.

Hawker, L. E., and P. M. Abbott. 1963. An electron microscope study of maturation and germination of sporangiospores of two species of Rhizopus. J. Gen. Microb. 32 : 295-298.

Ho, H. H. 1979. Electron microscope studies of oogonial wall and elemental composition of oogonia in Phytophythora. Mycopathologia, 68 : 17-21.

Hunsley, D., and J. H. Burnett. 1970. The ultrastructure of the walls of some hyphal fungi. J. Gen. Microbiol. 62 : 203-218.

Huwyler, H. R., Franz, G., and H. Meier. 1979. Changes in the composition of cotton fibre cell walls during development. Planta, 146 : 635-642.

Johnson, A. R. 1971. Improved method of hexosamine determination. Anal. Biochem. 44 : 628-635.

Kreger, D. R. 1954. Observations on cell walls of yeasts and some other fungi by x-ray diffraction and solubility tests. Biochim. Biophys. Acta, 13 : 1-9.

Lé John, H. B. 1974. Biochemical parameters of fungal phylogenetics. In : Evolutionary biology. Vol. 7. Edited by T. Dobzhansky, M. K. Hecht, and W. C. Steere. Plenum Press. N. Y. pp. 79-125.

Letourneau, D. R. 1978. Investigations on the host-parasite interface of a mycoparasitic system. Msc.Thesis. Brock University, St. Catharines, Ontario.

Letourneau, D. R., Deven, J. M., and M. S. Manocha. 1978. Structure and composition of the cell wall of Choanephora cucurbitarum. Can. J. Microbiol. 22 : 486-494.

Lilly, V. 1965. The chemical environment for fungal growth. In : The fungi.vol. 1. Edited by G. C. Ainsworth and A. Sussman. Plenum Press. N. Y. pp. 465-478.

Lilly, V., and H. L. Barnett. 1961. Physiology of the fungi. McGraw Hill Book Company. N. Y.

Lowry, D. H., Rosebrough, N. J., Farr, A. L., and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193 : 265-275.

Mahadevan, P. R., and E. L. Tatum. 1967. Localization of structural polymers in the cell wall of Neurospora crassa. J. Cell Biol. 35 : 295-302.

Mandels, G. 1965. Kinetics of fungal growth. In : The fungi. vol. 1. Edited by G. C. Ainsworth and A. Sussman. Plenum Press. N. Y. pp. 599-612.

Manocha, M. S., and J. R. Colvin. 1967. Structure and composition of the cell wall of Neurospora crassa. J. Bacteriol. 94 : 202-212.

Manocha, M. S., and J. R. Colvin. 1968. Structure of the cell wall of Pythium debaryanum. J. Bacteriol. 95 : 1140-1152.

Manocha, M. S., and R. Golesorkhi. 1979. Host-parasite relations in a mycoparasite. (V)Electron microscopy of Piptocephalis virginiana infection in compatible and incompatible hosts. Mycologia 71 : 565-576.

Manocha, M. S., and K. Y. Lee. 1971. Host-parasite relationships in a mycoparasite. (I) Fine structure of host, parasite and their interface. Can. J. Bot. 49 : 1677-1681.

Manocha, M. S., and K. Y. Lee. 1972. Host-parasite relations in a mycoparasite. (II) Incorporation of tritiated N-acetylglucosamine into Choanephora cucurbitarum infected with Piptocephalis virginiana. Can. J. Bot. 50 : 30-37.

Manocha, M. S., and D. R. Letourneau. 1978. Structure and composition of host-parasite interface in a mycoparasite system. Physiol. Plant Pathol. 12 : 151-157.

Marchant, H. J., and E. R. Hines. 1979. The role of microtubules and cell wall deposition in elongation of regenerating protoplasts of Mougeotia. Planta, 146 : 41-48.

McMurrough, I., Flores-Carreon, A., and S. Bartnicki-Garcia. 1971. Pathway of Chitin synthesis and cellular location of chitin synthesis in Mucor rouxii. J. Biol. Chem. 246 : 3999-4007.

McMurrough, I., and A. H. Rose. 1967. Effect of growth rate and substrate limitation on the composition and structure of the cell wall of Saccharomyces cerevisiae. Biochem. J. 105 : 189-203.

Mendenhall, W. 1971. Introduction to probability and statistics. Duxbury Press. USA.

Moore-Landecker, E. 1972. Fundamentals of the fungi. Prentice Hall Inc. N.Y.

Moreno, R. E., Kanetsuna, F., and L. M. Carbonell. 1969. Isolation of chitin and glucan from the cell wall of the yeast form of Paracoccidiodes brasiliensis. Arch. Biochem. Biophys. 130 : 212-217.

Morrall, P., and D. E. Briggs. 1978. Changes in cell wall polysaccharides of germinating barley grains. Phytochemistry 17 : 1495-1502.

Nickerson, W. J. 1963. Symposium on the biochemical basis of morphogenesis in fungi. (IV) Molecular basis of form in yeasts. Bacteriol. Rev. 27 : 305-324.

Potgieter, H. J., and M. Alexander. 1965. Polysaccharide components of Neurospora crassa hyphal walls. Can. J. Microbiol. 11 : 122-125.

Price, J. S., and R. Storck. 1975. Production, purification and characterisation of an extracellular chitosanase from Streptomyces. J. Bacteriol. 124: 1547-1585.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opague stain in electron microscopy. Cell Biol. 17 : 208-213.

Rosenberger, R. F. 1976. The cell wall. In : The filamentous fungi. Vol. 2. Edited by J. E. Smith and D. R. Berry. Edward Arnold Publishing Co. Ltd. USA. pp. 328-334.

Ruiz-Herrera, J., Sing, V. O., Van Der Woude, W. J., and S. Bartnicki-Garcia. 1975. Microfibril assembly by granules of chitin synthetase. Proc. Nat. Acad. Sci. 72 : 2706-2710.

Safe, S. 1974. Lipid and alkali extractable fatty acids from Mucor rouxii : Effect of thermal changes in growth environment and age of cells. Lipids, 9 : 952-956.

San-Blas, G., Ordaz, D., and F. J. Yegres. 1978. Histoplasma capsulatum. Chemical Sabouraudia, 16 : 279-284.

Sanger, V. K., and P. K. Dugan. 1973. Chemical composition of the cell wall of Smittium culisetae (Trichomycetes). Mycologia, 65 : 421-431.

Siestma, J. H., and J. G. H. Wessels. 1977. Chemical analysis of the hyphal wall of Schizophyllum commune. Biochim. Biophys. Acta, 496 : 225-239.

Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26 : 31-43.

Stadler, J. 1976. Quantitative analysis of total membrane-bound sugars as alditol acetates by combined thin-layer chromatography, gas-liquid chromatography and radiogas chromatography. Anal. Biochem. 74 : 62-72.

Storck, R., and J. S. Price. 1977. Sensibility of germinating spores of Mucor rouxii to chitosanase. Experimental Mycology, 1 : 323-338.

Tominga, Y., and Y. Tsujisaka. 1975. Purification and some enzymatic properties of the chitosanase from Bacillus R-4 which lysis Rhizopus cell walls. Biochim. Biophys. Acta, 410 : 145-155.

Tominga, Y., and Y. Tsujisaka. 1979. Degradation of cell walls of Mucor mucedo by chitosanase from Bacillus R-4. Agric. Biol. Chem. 43 : 2607-2609.

Umbreit, W. W., Burris, R. H., and J. F. Stauffer. 1964. Manometric techniques. A manual describing methods applicable to the study of tissue metabolism. Burgess Publishing Co. Minnesota.

Van Laere, A. J., Carlier, A. R., and J. A. Van Assche. 1977. Cell wall carbohydrates in Phycomyces blakesleeanus Burgeff. Arch. Microbiol. 112 : 303-306.

Wang, M. C., and S. Bartnicki-Garcia. 1970. Structure and composition of the cell walls of the yeast form of Verticillium albo-atrum. J. Gen. Microbiol. 64 : 41-54.

Weijman, A. C. M., and H. L. C. Meuzelaar. 1979. Biochemical contributions to the taxonomic status of Endogonaceae. Can. J. Bot. 57 : 284-291.

APPENDIX I

Sample Calculation for the Analyses of the Data by the Students t-Test

Initially, a number of assumptions must be fulfilled before the Students t-Test can be reliably applied to a specific experimental situation. These assumptions are as follows: (1) the sampled population possesses a normal probability distribution, and (2) the sample is randomly drawn from a normal population (Mendenhall, 1971). The manner in which the cell wall material was sampled appears to satisfy the latter assumption. However, the F-test must first be employed in order to assure the normality of the experimental data.

The experimental values collected from the extraction of protein from the fungal cell walls by 1N NaOH is used as an example in this calculation.

F-Test (Mendenhall, 1971)

$H_o : \sigma_1^2 = \sigma_2^2$

$$F = \frac{S_1^2}{S_1^2}$$

$$F = \frac{59.49}{34.71}$$

$$F = 1.71$$

At $F \alpha 0.5$, $F_{crit} = 5.05$, with $V_1 = 5$ and $V_2 = 5$.

$\therefore$ we do not reject H_o since there is insufficient evidence to indicate that there is a difference in the samples variances

Now, in order to determine whether or not the experimental means are significantly different, the Students t-Test is employed.

Students t-test (Mendenhall, 1971)

$$H_o: \mu_1 - \mu_2 = 0$$

$$H_a: \mu_1 - \mu_2 > 0$$

$$t = \frac{(\bar{Y}_1 - \bar{Y}_2)}{S\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

$$t = \frac{192.45 - 162.92}{6.86\sqrt{\frac{1}{6} + \frac{1}{6}}}$$

$$t = 7.45$$

At $t_{\alpha} 0.5$, $t_{crit} = 1.83$, with 10 degrees of freedom.

$\therefore$ since $t_{obs} > t_{crit}$, we will reject the null hypothesis and conclude that the protein concentrations of the 1 and 7 day old samples are significantly different.

The calculation of significant difference for the experimental data presented in this investigation followed the above formulae. In the instances where the variances of the means were significantly different (ie lipid composition of the cell walls), the assumptions of the Students t-Test could not be fulfilled. Therefore, the statistical analysis was not applicable to this data.

APPENDIX II

The Recovery Efficiency of the Sephadex G-10 Column

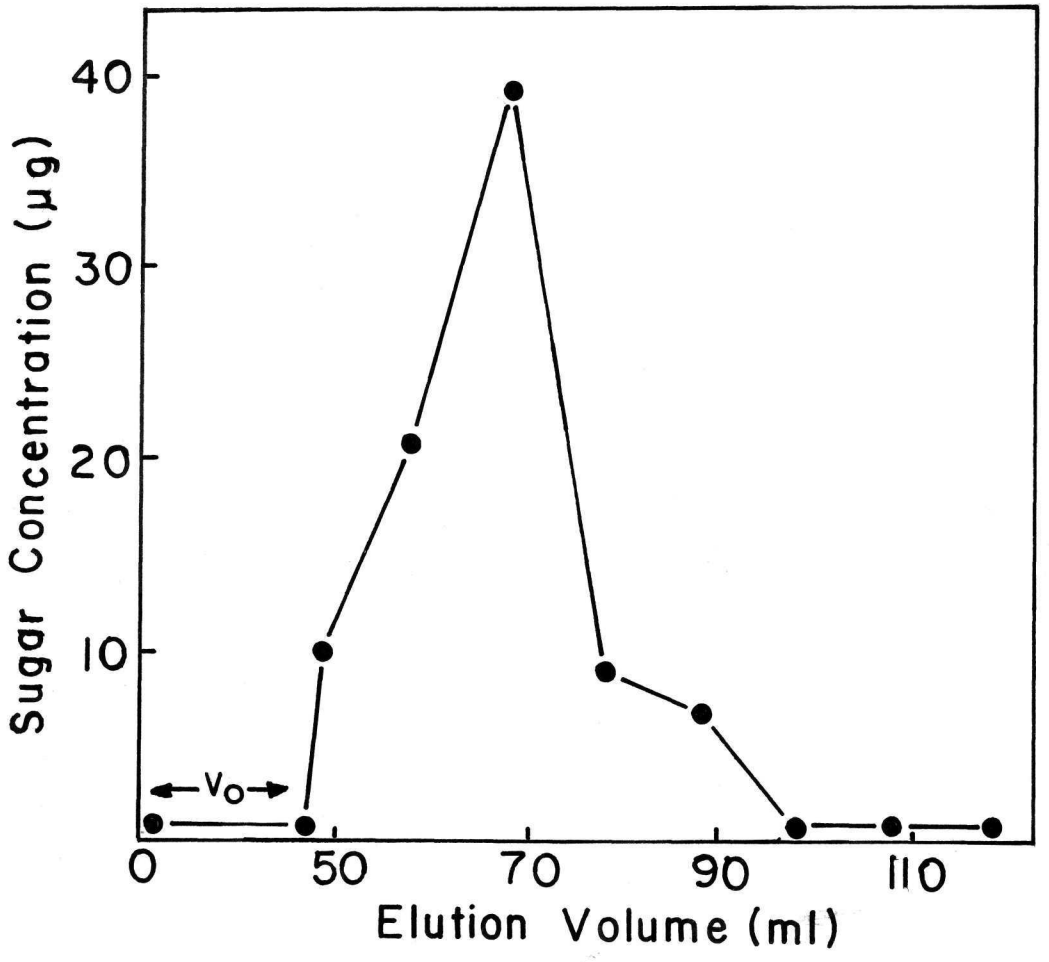
One hundred micrograms of glucose was pretreated with 5 ml of a 30% KOH solution (100°C, 1 hr.) and loaded onto a Sephadex G-10 column. The sugars were eluted with distilled water and aliquots from the column were reduced to a known volume *in vacuo*. The quantitative estimation for the concentration of glucose in each of these fractions was estimated by the Anthrone Method (Umbreit *et al.*, 1964.)

The data from this investigation reveals that 86% of the sugar which was loaded into the column was recovered within the first 88 ml of elutant (Figure 13). Beyond this point only trace amounts of sugars were detected. Similarly, there was a concurrent increase in pH which indicated that the salt solution began to elute from the column after approximately 108 ml of distilled water had passed through it.

The study which investigated the separation of uronic acids from the nitrous acid supernate utilizing the Sephadex G-10 column, was identical to that which is described above. However, a slight increase in the column's ability to recover the uronic acids from the effluent was noted.

Finally, the calculated efficiency of the column in each instance was incorporated into the data which is presented in Table III.

FIGURE 13: The recovery efficiency of the Sephadex G-10 column



NOTE: Vo = void volume of the column.